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CHEMICAL STUDIES
OF SOME HISTOLOGICAL COMPONENTS
OF WOOL

by

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FOR ALICE AND MONICA

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submitted for the degree of
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I hereby certify that except for work credited
FOR ALICE AND MONICA
to other people, all the work presented in this thesis is my own.

Samuel H. King

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SUMMARY

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Complete separation of exocuticle from endocuticle has been achieved by digesting the endocuticle with peracetic acid. The advice of his supervisor, Dr. J.H. Bradbury, and sincerely thanks the following: Dr. Bradbury's students, for providing much helpful discussion; Mr. D.E. Peters, for preparing thin sections for electron microscopy, and for operating the electron microscope; the staff of this Chemistry Department, for their willing assistance in numerous matters; Professor J.D. Smyth, formerly of the Botany Department of this University, for granting access to the electron microscope; Dr. W.L. Nicholas of that same Department, for advising Mr. Peters on techniques of electron microscopy; and the Australian Wool Board, for financial support. Finally, the author expresses gratitude for the patient encouragement and assistance received from his wife during this project.

A technique of aligning and embedding large numbers of cortical cells was developed. This allowed the confirmation by electron microscopy of the place of origin in the fibre, of light and heavy gold stained cortical cell fractions produced from gold stained fibre. Orthocortical cells were identified by the characteristic delineation of their microfibrils, and the light and heavy cell fractions were found to consist

S U M M A R Y

Complete separation of exocuticle from endocuticle has been achieved by digesting the endocuticle with pronase. Proof of the complete separation was obtained by determination of the percentage of cuticle dissolved at increasing periods of digestion, by comparison of the cuticle residue with undigested cuticle using electron microscopy, and by amino-acid analyses of the cuticle residue and the pronase digest. Hence the analyses represent the first amino-acid analyses performed of pure exocuticle and endocuticle.

Partial separation of exocuticle from endocuticle was achieved by preferential dissolution of exocuticle with performic acid. The percentage of cuticle digested at increasing periods of digestion was determined, and amino-acid analyses were performed on the performic digest and the performic residue. The amino-acid analyses of the performic digest and residue showed a number of similarities to the analyses of exocuticle and endocuticle respectively.

A technique of aligning and embedding large numbers of cortical cells was developed. This allowed the confirmation by electron microscopy of the place of origin in the fibre, of light and heavy gold stained cortical cell fractions produced from gold stained fibre. Orthocortical cells were identified by the characteristic delineation of their macrofibrils, and the light and heavy cell fractions were found to consist

CHAPTER I: INTRODUCTION

almost entirely of pure orthocortical and paracortical cells respectively.

The approach to equilibrium of the gold uptake of wool, stained under several conditions, was studied. This was correlated with light and electron microscopic evidence on the gold staining of fibres.

Further experiments were conducted on the desorption of gold from gold stained fibre using sulphuric acid, and on the gold staining of both unstained cortical cells and partially disrupted unstained fibre.

An analytical method was developed for the determination of gold in gold stained wool fibre and cortical cells.

The literature germane to exocuticle and endocuticle, and to the bilateral nature of the fine wool fibre, has been reviewed in this thesis.

Reduction of cuticle

1.1. Electron microscopy of cuticle residue obtained by pronase digestion

1.2. Amino-acid analyses

2. Discussion

2.1. Digestion of cuticle by pronase as a function of time

2.2. Electron microscopy of cuticle residue obtained by pronase digestion

2.3. Amino-acid analyses of cuticle

2.4. Amino-acid analyses of exocuticle and endocuticle

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C H A P T E R I

INTRODUCTION

The essential structural features of a fine wool fibre are displayed in Figure I-1. Such a fibre has a diameter of about 20 microns. The fibre has a well organised cellular structure, the cells of which have atrophied since the emergence of the fibre from the lower region of the follicle.

On the outside of the fibre is a thin sheath of flattened "cuticle" cells, which overlap each other or are "imbricated" in the manner shown. The extent of overlapping is small in fine wool fibres and is such that the cuticle sheath is usually only one or two cells thick (Bradbury and Leeder, 1970). Each cuticle cell has a stratified structure. An inner layer, the "endocuticle", comprises about half the thickness of the cell. Adjacent to and on the outer side of the endocuticle is another layer, the "exocuticle", which also has a thickness of about half that of the whole cell. The exocuticle is itself divided into the "a" layer and the remainder of the exocuticle, the "a" layer lying on the outer side. The thickness of the "a" layer is very approximately one-fifth that of the exocuticle. Each cuticle cell is completely surrounded by a cell membrane, and an "intercellular cement" lies between the membrane of adjacent cells. On the extreme outside surface of the fibre is a very thin layer, termed the "epicuticle", which is in fact that exposed outer part of the cuticle cell membrane which is not overlapped by adjacent cuticle cells (Leeder and Bradbury, 1971).

The cuticle sheath encloses the "cortex" which is comprised of longitudinally aligned spindle shaped "cortical" cells packed together. These cortical cells vary in length between roughly 50 and 100 microns, and are about 3 to 5 microns in width at their widest point. The ends of the cells are sometimes forked and the cells interdigitate with the adjacent cortical cells higher up and lower down the fibre. The degenerated nucleus, or "nuclear remnant", of the once living cortical cell is centrally placed within the cell and is about 10 microns long and 1 micron wide (Ryder and Stephenson, 1968). Part of, and surrounding, each cortical cell is a cell membrane, separated from the cell membranes of adjacent cortical cells by an intercellular cement. For both the cuticle and the cortex, in the region where any two cells abut, the cell membranes of the adjacent cells and the interleaved intercellular cement constitute a structural unit of constant width, and this unit is designated the "cell membrane complex".

As seen in Figure I-1 the cortex of a fine wool fibre is divided into two segments which lie on opposite sides of the fibre. These segments are called the "orthocortex" and the "paracortex", and the cells comprising them are called "orthocortical" and "paracortical" cells respectively.

Proceeding to lower levels of organisation we note that each cortical cell is subdivided into longitudinally aligned domains called "macrofibrils". This delineation into macrofibrils is not so well defined in paracortical as in orthocortical cells. The diameter of a macrofibril is about 0.2 micron. "Cytoplasmic debris" fills the spaces between macrofibrils. (See for example Rogers 1959a; Lundgren and Ward, 1963).

Macrofibrils themselves consist of longitudinally aligned "microfibrils" embedded in a "matrix" of amorphous protein. The diameter of a microfibril is about 80 angstroms (Rogers and Filshie, 1963).

There is evidence to suggest that each microfibril is composed of yet smaller components, the "protofibrils", which contain alpha-helical material (Filshie and Rogers, 1961).

All these components comprised of highly organised structures of individual molecules may be termed "histological components". Some authors prefer the description "morphological components".

The phenomenon of "bilateral differentiation", or subdivision of the cortex into two segments, is typical only of fine wool fibre and does not apply to the thicker fibres of coarse wools, to human hair, or to the various hairs, furs, and bristles of other animals. Chapman (1967) has summarised the arrangement within the cortex of these other types of keratin fibres. The "crimp" wave characteristic of the fine wool fibre is related to its bilateral differentiation.

The degree of overlapping of cuticle cells in these other fibres is much greater than in fine wool fibres and is often many cuticle cells thick.

The distinctive properties of wool and other keratin fibres are in part attributable to the extensive cross-linking between protein chains via the disulphide bonds in the frequently occurring cystine residue. In Chapter III we will refer to the separation of wool protein into a low-sulphur and a high-sulphur fraction (each fraction being a heterogeneous mixture of proteins) and so we indicate here the two methods of achieving this separation.

Reduction of the disulphides of wool with potassium thioglycollate in 6-8 M urea at pH 10-11, followed by alkylation of the free thiol groups with iodoacetate, yields a protein solution from which a low-sulphur fraction ("SCMKA", or "S-carboxymethylkerateine A") can be precipitated by adjusting the pH to 4.4, leaving the high-sulphur fraction ("SCMKB", or "S-carboxymethylkerateine B") in solution.

Treatment of wool with peracetic or performic acid (during which disulphides are oxidised to sulphonic acids) and then extracting the oxidised wool with ammonia solution, leaves an insoluble residue ("beta-keratose"), and yields a protein solution from which a low-sulphur fraction (alpha-keratose) can be precipitated by adjusting the pH to 4.4, leaving a high-sulphur fraction "gamma-keratose" in solution (e.g. Crewther et al. 1965).

SCMKA resembles alpha-keratose and both have a lower sulphur content than wool. SCMKB resembles gamma-keratose and both these have a higher sulphur content than wool.

The relatively small proportion by weight of the wool fibre occupied by cuticle (about 10%) belies the importance of this component. If wool be distinguished by its high sulphur content, then special consideration is due to the cuticle which is significantly richer in sulphur than the cortex (Bradbury, Chapman, and King, 1965). Though strength, extensibility, and elasticity of a single fibre are due mainly to the cortex, the mechanical properties of fabrics are determined by the frictional and adhesive forces between fibres, as well as by the mechanical properties of the fibres themselves. Owing to the imbricated surface, the conversion of wool fibres to fabrics is significantly affected by the greater resistance offered by the fibre to motion against the scale edges, than to motion in the opposite direction. Treatments which damage or modify the surface of the fibre can greatly affect the properties dependent on the nature of the surface. In important treatments affecting the cortex of the fibre, material must necessarily pass through the cuticle, often in both directions, and the cuticle is capable of having a controlling effect. Thus we now turn to separate investigations of the fine structure of two components of the fine wool fibre, the cuticle and the cortex, components which though discrete, are nevertheless intimately related.

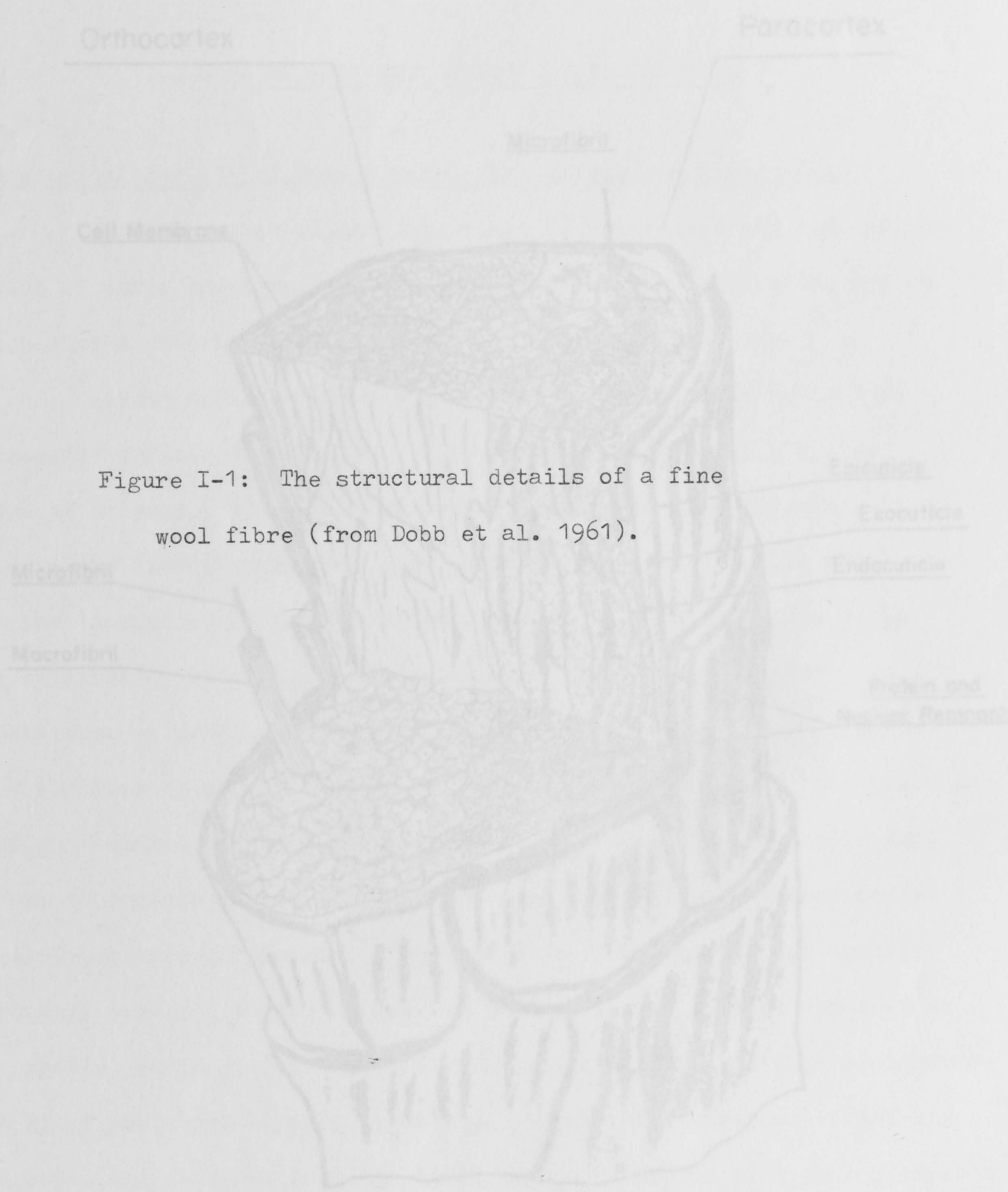
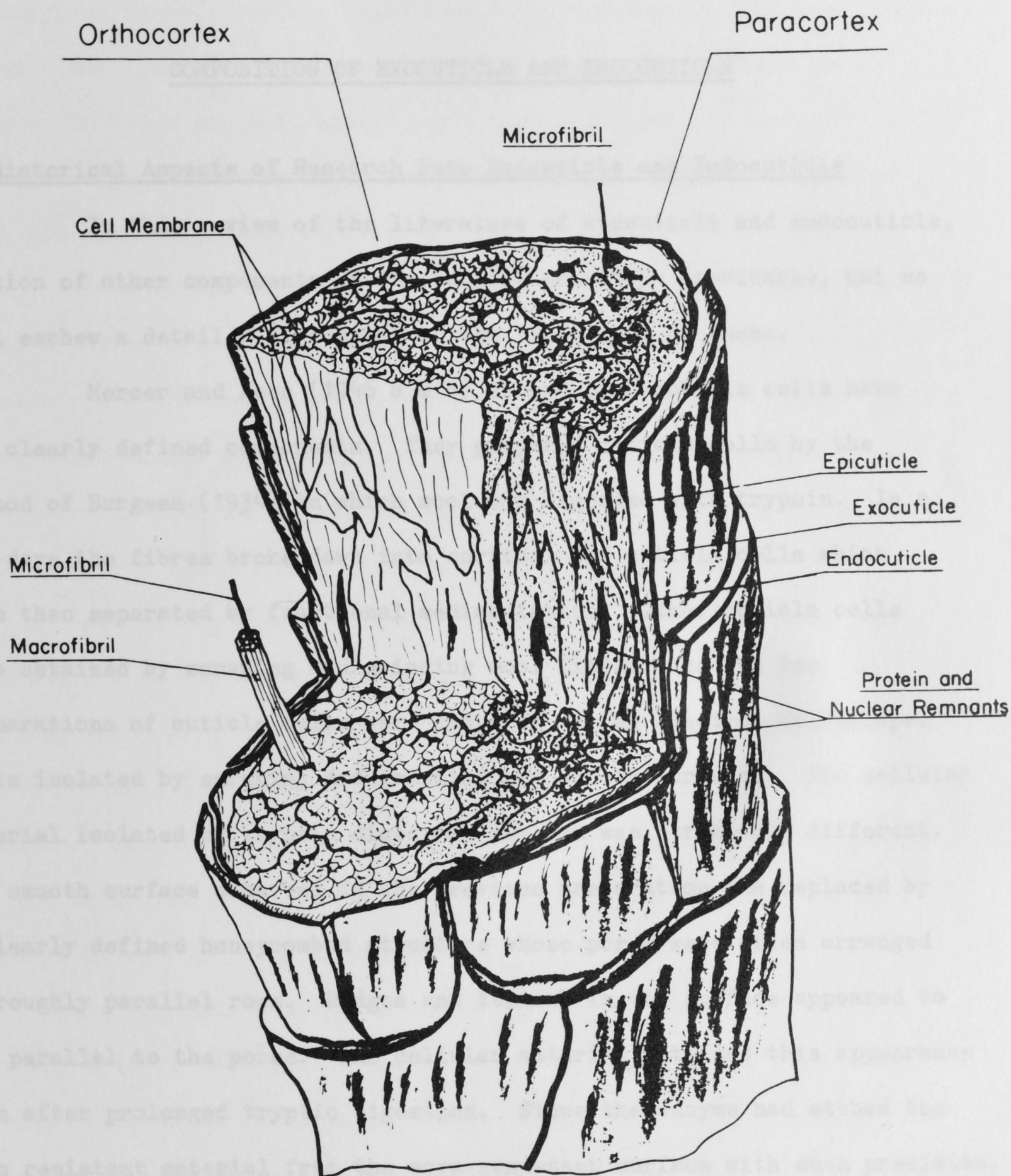


Figure I-1: The structural details of a fine wool fibre (from Dobb et al. 1961).



C H A P T E R I I

COMPOSITION OF EXOCUTICLE AND ENDOCUTICLE

1. Historical Aspects of Research into Exocuticle and Endocuticle

In this review of the literature of exocuticle and endocuticle, mention of other components of the cuticle sheath is inevitable, but we will eschew a detailed discussion of the literature of these.

Mercer and Rees (1946 a & b) showed that cuticle cells have two clearly defined components. They prepared cuticle cells by the method of Burgess (1934) in which wool was digested with trypsin. In a few days the fibres broke down into cortical and cuticle cells which were then separated by fractional sedimentation. Other cuticle cells were obtained by scraping and grinding wool fibres, and the two preparations of cuticle cells were compared in the electron microscope. Cells isolated by scraping and grinding had smooth surfaces. The cellular material isolated by tryptic digestion however was strikingly different. The smooth surface observed in the previous preparation was replaced by a clearly defined honeycombed structure whose pores were often arranged in roughly parallel rows. Ridges and furrows in the surface appeared to run parallel to the pores. The cellular material retained this appearance even after prolonged tryptic digestion. Since the enzyme had etched the less resistant material from the more resistant surface with such precision, and since the structure thereafter remained unaltered by weeks of exposure to the enzyme, it was concluded that cuticle has two clearly defined components.

Wool fibres were also treated with these reagents which dissolve keratin: sodium sulphide, caustic alkalis, halogens and sulphuryl chloride. Cuticle cells were then obtained by scraping these fibres. When examined in the electron microscope the surfaces of all these cells had a mottled, uneven appearance. Mercer and Rees thought that the reagents used had only partially digested away that component which had been completely removed by tryptic action.

Profiles of cuticle cells obtained by scraping from untreated fibres and from fibres treated with sulphuryl chloride were examined in the electron microscope. The re-entrant angle of the scale edge visible on untreated scales was missing on scales treated with sulphuryl chloride, and apparently had been either sealed over, or had been removed by the breaking off of the projecting scale tip of the cuticle cell.

Mercer and Rees concluded that the removal of the re-entrant angle was due to superficial attack on the cuticle cell by sulphuryl chloride, which partially digested the outer layer of the cuticle, and that it was this same layer which was completely removed by tryptic digestion. They assumed that the tryptic digestible layer " k_1 ", covered both the inner and outer surfaces of the cuticle and enclosed the more resistant material, " k_2 ", on the inside.

An electron micrograph due to Gorter and Houwink (1948 a & b) of spongy cuticle material obtained by tryptic digestion of Cape wool fibre can be seen in Lundgren and Ward (1963). The furrows, ridges, and regularity of pore arrangement observed by Mercer and Rees is not visible here. Like the earlier workers, Gorter and Houwink considered that the inner cuticle layer remains after tryptic digestion.

The outer and inner cuticle layers were soon named "exocuticle" and "endocuticle" respectively.

In reports at the time on epicuticle, the early conclusions above concerning the enzymic digestion of cuticle were accepted, and the exocuticle was thought to be a protein less extensively cross-linked than the inner, more enzyme-resistant endocuticle (see for example Lindberg, Mercer, Philip, and Gralen, 1949; Mercer, Lindberg, and Philip, 1949; Gralen, 1950; Fraser and Rogers 1955 b; Ramanathan, Sikorski, and Woods, 1955 a & b).

The introduction of the technique of thin sectioning, together with heavy metal staining, allowed the preparation of electron micrographs of both transverse and longitudinal sections of keratin fibres, especially in the region of the follicle (Farrant and Rogers, 1954; Barnicet, Birbeck, and Cuckow, 1955; Mercer, Farrant, and Rees, 1955; Birbeck and Mercer, 1957; Challice and Sikorski, 1957).

The specimens in the excellent micrographs of Birbeck and Mercer (1957) were stained with osmium tetroxide. We first consider their sections through the human hair follicle. A longitudinal section through the upper bulb region of the follicle shows a vertical row of cuticle cells with the cortex on one side and the inner root sheath on the other. The cuticle cells at the bottom of the row are cuboidal; those at the top have become thinner and slightly imbricated. The nuclei of the cuticle are near the cortical side of the cells. A section through the extremely imbricated cuticle cells found at the level where the cells are beginning to synthesise keratin, show small dark drops of keratin forming near the sheath side of the cells. In the cytoplasm are numerous granules, a few mitochondria, and also the nucleus which is visible at the bottom of the cell and on the cortical side. In a section of cuticle at the level where the cells are partly keratinised, large dark bodies of keratin on the sheath side can be distinguished from the much lighter and extremely granular cytoplasm. A section of completely keratinised cuticle cells shows almost no difference in transmission across the width of each cell. The cytoplasm has condensed,

and while not as granular as in the previous micrograph, is still visibly more granular than the keratin which lies on the outside of each cell. The degenerated nucleus can be seen, forced towards the cortical side of the cell by the developing keratin.

Micrographs of the following sections of material subjected to various treatments are also presented. A cuticle cell isolated by tryptic digestion shows that the condensed cytoplasmic region has been eaten away. The keratinised region has not been attacked and on the outside is a thin dense layer which is also resistant. A wool fibre previously oxidised with peracetic acid and extracted with ammonia shows intact the condensed cytoplasmic region of the cuticle. The keratinised region of the cuticle has been extracted and the leading edge of the cuticle appears as a thin dense line which has resisted attack. Peracetic acid followed by ammonia extraction was used because this treatment had been employed by other workers to break down keratin fibres (Alexander and Earland, 1950 a & b; Golden, 1954; Golden, Whitwell, and Mercer, 1955). Human hair cuticle fixed in 1-2% osmium tetroxide for a prolonged period of 48 hours shows the resistant leading edge of the cuticle to be heavily stained, and the keratinised region is stained slightly darker than the cytoplasmic region.

Birbeck and Mercer concluded that keratinisation of cuticle occurs at a late stage of its development, the keratin formed being of an amorphous type similar to the gamma fraction of the cortex which is produced at a similar level. In the final stage of keratinisation the remaining cytoplasm condenses, leaving the cell with a laminated structure, the outer layer of which is keratinised.

We note that these results necessarily reverse the interpretation put upon the early experiments of tryptic digestion of cuticle (Mercer and Rees, 1946 a & b; Gorter and Houwink, 1948 a & b). It is the highly cross-linked outside exocuticle layer which remains after tryptic digestion.

The reagents sodium sulphide, caustic alkalis, halogens and sulphuryl chloride used by Mercer and Rees probably did degrade the exocuticle, just as they believed, thereby accounting for the disappearance of the scale re-entrant angle with sulphuryl chloride. As the mottled appearance of the cuticle residues obtained with these reagents was slightly similar to the more clearly etched cuticle tryptic residue, they concluded that the exocuticle was degraded by trypsin as well. Lundgren and Ward (1963) have missed the significance that Birbeck and Mercer's work holds for deciding which cuticle component is digested by enzymes. They cite the evidence of only the early literature and accept the conclusion that the endocuticle is the resistant component.

The existence of a separate component of the exocuticle, the "a" layer, about 0.1 micron thick and lying just below the epicuticle, has been often observed (Lagermalm, 1954; Fraser and Rogers, 1955 a & b; Birbeck and Mercer, 1957; Sikorski and Simpson, 1958; Kassenbeck, 1959; Rogers, 1959 a; Sikorski and Simpson, 1959; Sikorski, Simpson, and Woods, 1960; Dobb, Johnston, Nott, Oster, Sikorski, and Simpson, 1961; Bradbury, Rogers, and Filshie, 1963; Lundgren and Ward, 1963; Bradbury and Chapman, 1964; Leach, Rogers, and Filshie, 1964; Swift, 1965; Swift and Holmes, 1965; Piper, 1966; Swift, 1968).

The "a" layer is known to be resistant to enzymes since it is visible as a densely stained band on the outside of cuticle isolated by tryptic digestion (Birbeck and Mercer, 1957).

In fibres oxidised with peracetic acid and then extracted with ammoniacal or alkaline buffer, the exocuticle is dissolved and the very outside limit of the cuticle appears dark in electron micrographs (e.g. Birbeck and Mercer, 1957; Rogers, 1959 b). This dark band is the "a" layer which is therefore held to be insoluble in reagents which dissolve keratin (e.g. Sikorski and Simpson, 1958; Dobb et al., 1961).

This is surprising for there is abundant evidence that the "a" layer is rich in sulphur. Osmium tetroxide staining of cuticle which has been reduced with thioglycollic acid always shows a very heavy deposit of metal in the "a" layer. The heavy staining of the "a" layer contrasts sharply with the moderate staining of the rest of the exocuticle, which in turn contrasts slightly but noticeably with the less heavy staining of the endocuticle. Rogers (1959b) found that reduced wool takes up much more osmium than can be accounted for by the reaction of the osmium tetroxide with sulphhydryl groups. It seems that fission of disulphide bonds also makes the structure more accessible to the osmium tetroxide. Now if cuticle is not reduced, a much longer time is required to stain the "a" layer intensely with osmium (Birbeck and Mercer, 1957). Thus intense staining of the "a" layer in reduced cuticle is evidence for the presence of large amounts of cystine in unmodified cuticle. Prolonged treatment in 1-2% osmium tetroxide for 48 hours, without prior reduction of disulphide bonds, stains the "a" layer of human hair cuticle intensely; the rest of the exocuticle is only moderately stained, but is nevertheless slightly more stained than the endocuticle (Birbeck and Mercer, 1957). Now the moderately stained region of the exocuticle both contains cystine (it is enzyme-resistant) and stains more deeply than the endocuticle. Therefore the much greater difference in staining between the "a" layer and the rest of the exocuticle reflects a great difference in cystine content.

Again, the presence of sulphur in the "a" layer has been nicely demonstrated by Sikorski and Simpson (1958), Rogers (1959a), and Swift, (1968). Using sodium plumbite staining at pH 11.8, the first group obtained dark spots of lead sulphide over the "a" layer in photomicrographs of the cuticle of Merino and Lincoln wools, mohair fibre, human hair, pig bristle and porcupine quill. The presence of lead sulphide in the fibres was confirmed by X-ray diffraction.

Rogers (1959a) coupled the sulphhydryl groups of reduced Lincoln wool with methyl mercuric iodide. Under the action of the electron beam of the electron microscope, there rapidly appeared in the cuticle and cortex a dense granulation, attributed to the decomposition of the $-SHgCH_3$ groups to resolvable particles of HgS. The endocuticle was almost free of granules, this showing that it is nonkeratinous. Dense accumulations of granules were seen in the exocuticle, while especially heavy deposits formed an extremely dense outer layer about 0.1 micron thick similar to the region which stains with osmium tetroxide. Since methyl mercuric iodide is specific for cysteine, the distribution of granules approximated the distribution of sulphur.

Swift (1968) used silver-methenamine reagent (a buffered solution of silver nitrate and hexamethylene tetramine) at alkaline pH to demonstrate the presence of cystine in the "a" layer and the rest of the exocuticle of human hair. Prolonged treatment with this reagent resulted in a very heavy deposition of metallic silver particles in the "a" layer and a somewhat lower concentration of particles in the rest of the exocuticle. No particles were deposited in the endocuticle. Conversion of the cystine of the hair sections to S-carboxymethyl cysteine prevented the deposition of silver particles by the reagent. This is strong evidence that the presence of the particles implies the presence of cystine. The use of the reagent for shorter periods stained the "a" layer and the rest of the exocuticle, but not the endocuticle, with the same relative intensity as before, but no metallic particles were deposited. Thus this "morphological" staining also reflects the distribution of cystine. This morphological staining was also visible in the treatments at the longer periods, and after both long and short periods of treatment could be removed by thiosulphate solution. The metallic particles were not removed by thiosulphate, and they were therefore composed of elemental

silver. Swift did not consider that the distribution of metallic silver reflected merely the relative accessibilities of the components, since the sections he treated were thin and were also probably swollen under the alkaline conditions used. We recall the remark of Rogers (1959b) that accessibility, as well as sulphur content, was a factor in determining the uptake of osmium by reduced wool. With osmium, sectioning has usually been performed after staining, not before, as was the case with Swift's experiments.

Rogers (1959a) used methylene blue to stain cross sections of wool follicles which had been oxidised with peracetic acid. This procedure is known to stain keratin intensely, and under the light microscope the exocuticle of the developing cuticle was similarly intensely stained. Thus the presence of cystine in exocuticle is implied.

Diffusion of the silver ammonium ion into human hair was investigated by Swift (1965). For untreated hair an electron micrograph and a microdensitometer trace of that micrograph are presented, and show a falling off in optical density across the layers of several adjoining cuticle cells due to the progress of the ion into the fibre. However, this decline is not even, and the trace exhibits a number of peaks corresponding to the various morphological components of each cuticle cell. This feature can also be observed in the micrograph itself; the "a" layer, the rest of the exocuticle, and the endocuticle are reasonably well defined. Not only has diffusion of the ion into the fibre occurred, but the ion has also reacted preferentially with certain of the components during its progress towards the cortex. This explains the peaks in the trace and the delineation of the "a" layer, the rest of the exocuticle, and the endocuticle in the micrograph.

Intense staining of the endocuticle in thin sections of wool fibres post-stained with phosphotungstic acid has been observed in the electron microscope (Bones and Sikorski, 1967). The very large diameter

of the phosphotungstic acid molecule (12 angstroms) would prevent the penetration of this reagent into highly cross-linked material, and so cross-linking in exocuticle is implied by the above evidence.

Swift (1965) could not stain untreated whole fibres of human hair with phosphotungstic acid. In contrast to the staining of endocuticle in thin sections, a dye molecule can only reach the endocuticle of an intact fibre by diffusion through the epicuticle and the highly cross-linked exocuticle. Hence the large phosphotungstic acid molecule could not stain the endocuticle in this case.

Extraction of wool with boiling hydrochloric acid at pH2 for 96 hours completely extracted the endocuticle (Leach, Rogers, and Filshie, 1964). Since this treatment is known not to attack disulphide bonds the result infers the presence of cystine in exocuticle.

Some of the cuticle prepared by ultrasonication of wool fibre in formic acid for 30 minutes (Bradbury and Chapman, 1964) showed signs of erosion in the endocuticle. The greater resistance of exocuticle to the mechanically disruptive influence of ultrasonics can be explained by the presence of cross-links in exocuticle.

Alexander, Hudson, and Earland (1963) have discussed the dissolution of wool by enzymes. The digestion of whole wool fibres by proteolytic enzymes is enhanced by prior treatment with reagents which attack cystine.

Finding that he had completely dissolved the interior of the fibre, Geiger (1944 a, b & c) considered that he had isolated intact scale material by digesting reduced and ethylated wool with pepsin. The residue consisted essentially of protein and contained very large amounts of sulphur. One presumes the pepsin digested the endocuticle. If the exocuticle had been completely reduced, then it too should have been digested, yet the residue was rich in sulphur. Reduction sufficient to give complete dissolution of the cortex with pepsin could still leave the much more

sulphur-rich exocuticle substantially unreduced. The other possibility is that the residue was largely epicuticle, since King and Bradbury (1968) found that epicuticle contained only slightly less cystine than whole cuticle. Epicuticle is known to be resistant to enzymes; (e.g. Leeder, 1969).

Dissolution of wool with peracetic acid, ammonia, and then trypsin furnished Golden, Whitwell, and Mercer (1955) with undissolved membranes which King and Bradbury (1968) believe to be no more than a very impure sample of epicuticle. In view of experiments previously mentioned it is likely that the exocuticle was dissolved by the extraction with ammonia after peracetic oxidation, and that the trypsin dissolved the endocuticle.

With a solution of papain and bisulphite Holmes (1964) isolated cuticle material from human hair as thin curled up plates. These lack the more substantial appearance of cuticle isolated by mechanical disruption of whole fibre in formic acid (Bradbury and Chapman, 1964). If the bisulphite had reduced all the exocuticle, then the papain would have dissolved both exocuticle and endocuticle. Holmes however considered that the hair was only partially reduced, and that the sulphydryl groups produced activated the papain, since in a papain digestion slowed by the prior conversion of cystine of the wool substrate to lanthionine (which is not reduced by bisulphite) the addition of cysteine restored the rate of digestion to normal.

Swift and Holmes later showed by electron microscopy (1965) that though it extracted endocuticle from human hair, the papain and bisulphite reagent did in fact leave the exocuticle intact.

Leeder (1969) extracted roughly 20% of the weight of cuticle with trypsin and papain each at pH 8.4 and 40°C for one week. With pepsin at pH 1.2 and 40°C for ten days he extracted 26%. The weight extracted with trypsin was the same after one, four or seven days. Amino-acid analysis

revealed no cystine in the tryptic digest and about as much cystine in the tryptic residue as in his sample of whole cuticle. He concluded that he had extracted at least part of the endocuticle.

The dissolution Leeder achieved with papain and pepsin no doubt reflects the specificity of these enzymes. Pepsin splits a peptide bond derived from the amino group of phenylalanine or tyrosine (Fieser and Fieser, 1961). Though Swift and Holmes (1965) apparently achieved complete dissolution of endocuticle with papain, they used bisulphite in conjunction with the enzyme.

The early investigations of Woods (1938) and Rudall (1941) using X-rays and birefringence, and the electron microscopy of Hock and McMurdie (1943 a & b) showed that cuticle cells contained amorphous protein whereas the cortex contained regular fibrillar structures. A fragment of the tryptic cuticle residue of Mercer and Rees (1946 a) was seen torn obliquely to the furrows of the residue, and so these workers thought that the alignment of the furrows, ridges, and pores of the residue was not due to the alignment of the molecules of the structure.

More recently Haly, Snaith, and Anderson, (1970) have examined the surface of a mechanically lacerated monkey hair in the scanning electron microscope. They remark upon the lack of evidence of a fibrous nature in cuticle. No broken fibrils were seen protruding from the cuticle, and a tear in the cuticle had travelled quite readily in both the axial and transverse directions.

Nevertheless there is some evidence supporting the existence of fibrils in cuticle as we shall now see.

In isolated cuticle cells degraded with pancreatin, fibrils similar to those of the cortex were observed (Zahn, 1943).

Fraser and Rogers (1955 a) presented evidence, which they claimed, implied the existence of a longitudinally aligned fibrillar component of endocuticle, embedded in a less resistant endo cementing substance. Yet

their interpretation of their results is complicated by their belief at that time that endocuticle was resistant to tryptic digestion.

Happey and Johnson (1962 & 1965) have reported electron microscopic evidence for the development of fibrils at the boundary between the exo- and endocuticle in cuticle from the follicle region of human hair.

The epicuticle is now known to be a very thin (30-100 angstroms) chemically resistant membrane, found on the extreme outer surface of the fibre, and for any given cuticle cell this membrane covers that exposed portion of the exocuticle which is not overlapped by the adjacent external cuticle cell. But this membrane also completely surrounds each cuticle cell and hence is part of the cell membrane complex lying between overlapping cuticle cell layers (Leeder, 1969; Leeder and Bradbury, 1971).

Its presence inferred by Muller (1939) and Whewell and Woods (1944), the epicuticle was first isolated by Lindberg, Philip, and Gralen (1948). Sacs appearing on fibres treated with chlorine water (Allworden 1916) and bromine water (Herbig 1919) are produced by dissolution of material underlying the epicuticle (Ames 1952; Leveau, Langlois and Parisot 1952; Schuringa, Konings, and Ultee 1953; Fraser and Rogers 1955 b; King and Bradbury 1967; Leeder 1969; Bradbury and Leeder, 1971). The nature of the immediately underlying layers of exo- and endocuticle therefore bears directly on the production of these sacs. In the case of the Herbig sacs produced by bromine water, the epicuticle is thickened by adhering exocuticle (Fraser and Rogers, 1955 b; King and Bradbury, 1968). With mammalian hair, underlying material is often raised with the epicuticle and this occurs more readily with bromine than chlorine. Bromine water actually lifts the entire cuticle of Lincoln wool and some other coarse fibres (Leveau, Langlois, and Parisot, 1952; Fraser and Rogers, 1955 b). The contents of Allworden and Herbig sacs, and the membranes raised above them, have been analysed for their amino-acid content by King and Bradbury (1968). The mechanisms of Allworden and Herbig sac formation have since been intensively investigated (Leeder, 1969; Bradbury and Leeder, 1971).

2. Materials and Methods

2(a). Cleaning of wool

Merino 64's fibres with their tips removed were cleaned by Soxhlet extraction with petroleum (b.p. 40-60°C) for four hours (Bradbury and Chapman, 1964). They were washed in water at less than 60°C until further washing caused no further discolouration of the water, and were then left to dry in the air.

2(b). Preparation of Cuticle

The fibres were cut with scissors into lengths of about 0.25 cm. Skin flakes were removed from the fibres by agitating these short lengths in 99% formic acid in a Vibromix agitator obtained from A.G. fur Chemie-Apparatebau, Zurich (Bradbury, Chapman, Hambly, and King, 1966).

The fibres were then reduced to their cellular components by chopping in 99% formic acid with a Polytron cutter (available from Kinematica GMBH). Since formic acid dissolves intercellular material, chopping for very short periods released from the fibre vast quantities of intact cortical cells, large fragments of cuticle cells, and only relatively small amounts of finely chopped cortical and cuticle cells. (Prolonged chopping for 45 minutes reduces all the fibres to very fine cuticle and cortical fragments).

Cuticle was produced in batches. For each batch a 12 gm sample of wool was subjected to twenty successive operations: in each operation the wool was chopped for 10 seconds with the Polytron, the particles produced were collected by filtration through a coarse wire sieve, and the retained fibrous mass was returned to the Polytron vessel. The formic acid was removed from the particles by spinning them down in the centrifuge and washing four times with ethanol.

Particles longer than 50 microns, namely small pieces of fibre, clusters of cortical cells, and most individual cortical cells, were

removed by filtration through a 50 micron Nytrel nylon filter cloth as follows. After redispersion of aggregated particles by ultrasonication for 10 seconds, the suspension was filtered, and the retained material was re-suspended in ethanol, ultrasonicated, and filtered again. The two filtrates were combined.

Cortical cells which had gone end on through the Nytrel filter were removed by six filtrations of the suspension through an 18 micron Endicott metal sieve, the material retained being discarded each time.

A large quantity of tiny cortical fragments, and tiny cuticle fragments, were removed by spinning in a small bench top centrifuge for 3 minutes and discarding the supernatant. Ten such operations were required, the sedimented material being re-suspended in ethanol each time. The ten operations reduced the contamination by the tiny cortical fragments to an acceptable level.

Though a particle count under the light microscope revealed that one particle in twenty was a cortical fragment, the contamination of cuticle by cortical material was much less than 5% by weight since each cortical particle was very small and very thin, whereas the cuticle particles were large flat fragments of cuticle cells.

The cuticle was transferred to water at the centrifuge, thereby replacing ethanol in the interior of the keratin with water. The sample was dried in vacuo. In one batch an initial 12 gm of wool gave about 200 mg of cuticle - a yield of 1.7%.

2(c). Pronase digestion of cuticle

Pronase was obtained from K. and K. Laboratories. The conditions of enzymic treatment were adapted from those used for whole wool by Springell (1963).

The digestions were performed in small bottles of slightly greater capacity than 4.0 ml and which were immersed in a water bath at 37°C. Each bottle was capped with a piece of polythene sheet underneath its screw-cap,

and was vigorously shaken at intervals during the digestion.

Each vessel contained the following: about 22 mg of cuticle accurately weighed (21.97 mg of cuticle weighed at room conditions had been found to have a dry weight of 20.00 mg); 3.6 ml of 1% w/w ammonium acetate buffer at pH 8.0 containing 10% v/v ethanol; and 0.4 ml aqueous pronase solution containing either 1 mg or 0.2 mg of pronase. The cuticle to pronase ratios used were therefore 20 : 1 and 100 : 1 by weight. An additional amount of pronase was added to one sample during digestion to check whether autolysis of pronase was occurring.

After the required time of digestion the contents of each bottle were filtered through a preweighed sintered glass crucible having a layer of Gooch asbestos filter-aid on the sinter. (Filtration is impossible without the filter-aid). The crucibles were dried in vacuo and weighed.

The percentage of cuticle dissolved was plotted against time of digestion for both the 20 : 1 and 100 : 1 cuticle to pronase ratios.

In several cases, after reaction the residue was separated from the digest by sedimentation in an ultracentrifuge. After removal of the supernatant the sediment was washed with copious quantities of water to remove all traces of the digest, then dried and set aside for later amino-acid analysis and examination in the electron microscope. Ammonium acetate, being volatile, was separated from the digest by rotary evaporation of the supernatant. Water was added before reaching dryness and the evaporation continued. It had been found that no ammonium acetate residue remained after rotary evaporation of 3.6 ml of the buffer six times, water being added each time before dryness was reached. Six such cycles were therefore used on the solution of digest. Lyophilisation was finally used to try to dry the sample completely, but it could only be obtained as a damp viscous gum. It was set aside for amino-acid analysis.

2(d). Digestion of cuticle with urea/thioglycollic acid

Digestions of cuticle at 25°C were performed in 10 ml volumetric flasks immersed in a water bath. Each flask contained 20 mg (dry weight) of cuticle and 5.0 ml of urea/TGA solution which was 8M in urea, 0.2M in TGA, and adjusted to pH 11 with NaOH. After reaction the contents of the flask were filtered in the manner used for the enzymic digestions and the percentage of cuticle dissolved was obtained.

2(e). Oxidation of cuticle with performic acid

Nine volumes of 99% w/w formic acid were mixed with one volume of 30% w/v hydrogen peroxide and the resultant performic acid mixture used two hours later (Toennies and Homiller, 1942).

Oxidations were performed in 5 ml volumetric flasks immersed in a water bath at 37°C. Each flask contained about 20 mg (dry weight) of cuticle accurately weighed and 5.0 ml. of performic acid mixture. The flasks were shaken from time to time and after reaction the contents of each were filtered and weighed as previously described for pronase digestion (Section 2(c)). The percentage of cuticle dissolved was plotted against time of reaction. After one digestion the performic residue and digest were separated and dried as described for the pronase residue and digest.

The stability of performic acid at 37°C was studied by the method of Toennies and Homiller (1942). To 20 ml of 0.5 M KI was added 1 ml of performic acid mixture and the iodine liberated was titrated with thiosulphate, starch indicator being added just before the end-point. The percentage of performic acid decomposed was plotted against time of decomposition.

2(f). Electron microscopy

Cuticle residues were reduced with 0.5 M thioglycollic acid at pH 5.6 and stained with osmium tetroxide (Rogers, 1959 b). They were then embedded in Araldite (Glauert, Rogers and Glauert, 1956). The Araldite ingredients were obtained from CIBA Ltd. and the following recipe was used:

10 gm Resin M; 10 gm Hardener HY 964; 1 gm Accelerator; and 20 drops dibutyl phthalate. Each sample was transferred in three stages to propylene oxide solutions of progressively increasing Araldite content until the last solution contained no propylene oxide. Accelerator was present only in this last solution which was centrifuged in a small gelatin capsule to concentrate the sample before curing overnight at 60°C.

The author is especially indebted to a colleague, Mr. D.E. Peters, who cut thin sections from Araldite blocks of embedded cuticle residue with an L.K.B. Ultratome, post-stained these sections, and operated the Hitachi Model HU-11C-S electron microscope.

2(g.) Amino-acid analysis

A Technicon double column amino-acid analyser was used. Internal standards of taurine, norleucine and alpha-amino-beta-guanidinopropionic acid were added to each sample prior to analysis (Bradbury, Chapman, and King, 1965). Before loading on the columns, each sample was hydrolysed with 6M HCl at 110°C for 24 hours, and then dissolved in citrate buffer.

For each point the performic acid solution was used two hours after making the constituent formic acid and a known amount of taurine.

2(h.) Reduction of cuticle

Preliminary studies of the reduction of 27% of cuticle with thioglycollate, in the presence of urea, at pH 2.4 were attempted but later abandoned owing to experimental difficulties. The following data were obtained:

Time of reaction in hours:	0.5	1.0	2.0	4.0	8.0	16.0
Percentage dissolved:	13.3	17.2	17.2	15.5	13.8	10.4

Only the first two entries in this table correspond to samples for which

Gosch asbestos was used in the sintered glass crucibles during filtration.

The filtration of the remaining samples took only hours and so the actual

time of reaction is uncertain. Nevertheless the effect of dissolution

achieved is quite substantial and presumably partial degradation of the cuticle occurred.

3. Results

3(a). Digestion of cuticle by pronase as a function of time

Figure II-1 shows the progress with time of the pronase digestion of cuticle. Included in the plateau region of the curve for the digestion using a cuticle to pronase ratio of 20 : 1, is a point corresponding to the percentage of cuticle digested in a case where an additional amount of pronase was added to the reaction halfway through the digestion, making the cuticle to pronase ratio 10 : 1.

The first digestion performed was for one day and the low value obtained was due to experimental difficulties in developing the method. The aim was to determine the maximum dissolution and so the approach to equilibrium was not investigated in detail. Therefore the first digestion was not repeated, and further digestions of up to four weeks using a ratio of 100 : 1 were not necessary.

3(b). Odixation of cuticle

Figure II-2 shows the progress with time of both the dissolution of cuticle by performic acid, and the decomposition of performic acid. For each point the performic acid solution was used two hours after mixing the constituent formic acid and hydrogen peroxide.

3(c). Reduction of cuticle

Preliminary studies of the dissolution at 25°C of cuticle with thioglycollate, in the presence of urea, at pH 11 were attempted but later abandoned owing to experimental difficulties. The following data were obtained:

Time of reaction in hours:	0.25	0.5	0.5	1	2	4
Percentage dissolved:	13.3	17.2	17.2	15.5	19.8	18.4

Only the first two entries in this table correspond to samples for which Gooch asbestos was used in the sintered glass crucibles during filtration. The filtration of the remaining samples took many hours and so the actual time of reaction is uncertain. Nevertheless the extent of dissolution achieved is quite substantial and presumably partial degradation of the endocuticle occurred.

3(d). Electron microscopy of cuticle residue obtained by pronase digestion.

Figures II-3 and II-4 depict sections through cuticle residues obtained from digestions at 37°C using a cuticle to pronase ratio of 20 : 1 for one week's digestion. Figure II-5, inserted for comparison and taken from Bradbury and Chapman (1964), shows a section through an unmodified cuticle fragment. The fragment was produced by ultrasonication of whole fibre in formic acid and the section was post-stained with lead hydroxide.

3(e). Amino-acid analyses

Data relating to the samples analysed appear in Table II-1. Cuticle samples A and B were taken from separately prepared batches. Residues and digests from pronase action on cuticle are designated "Exo" and "Endo" respectively. The analysis of Exo A was obtained using only one column of the amino-acid analyser. The analyses of all the other samples were obtained using both columns, and so each analysis is the mean of two analyses. Exo A and Endo A were separated after digestion of the one original cuticle sample. Likewise Exo C and Endo D originate from the same sample. In the case of Endo C more pronase was added after two weeks of the reaction, which was then allowed to continue for another two weeks. As each Endo sample could only be obtained as a damp gum (Section 2c), it was impossible to obtain accurate dry weights of these samples for analysis. This accounts for the low recovery of anhydroamino-acids in the case of the Endo samples.

The analyses are presented in Tables II-2 to II-7.

In Table II-2 the analysis of Cuticle C (King and Bradbury, 1968) is the mean of analyses of three different cuticle samples produced by ultrasonication of whole fibre in formic acid, dichloroacetic acid, and dimethyl sulphoxide respectively. (See also Bradbury, Chapman, and King, 1965). Samples were not analysed for citrulline in the present work.

Since pronase digestions using cuticle to pronase ratios of 50 : 1 and 100 : 1 did not quite attain maximum dissolution, the Exo C sample was slightly contaminated with endocuticle. Exo C comprises 69% of the original weight of cuticle whereas the pure exocuticle in Exo C is only 64% of the original weight. As this contamination is small and since the analyses of the three Exo samples vary anyway, the analysis of Exo C in Table II-3 can be regarded as the analysis of pure exocuticle.

It was necessary to correct the analyses of the digests obtained by pronase action on cuticle, for the amount of pronase contained by the digests, and so an analysis of pronase was performed (Table II-4). The recovery of anhydroamino-acids in this case was only 48.2% owing to the loss of an unknown amount of material during the preparation for analysis, but the validity of the analysis was not affected. In Table II-4, Endo A and Endo B (cuticle to pronase ratio of 20 : 1) contain more pronase than Endo C (50 : 1) which in turn contains more pronase than Endo D (100 : 1). Pronase is significantly richer than the digests containing the most pronase, Endo A and Endo B, in glycine, histidine, alanine, threonine, aspartic acid, and tyrosine in that order. The corrections for these amino-acids constitute between 6% and 10% of the final corrected values. The corrected analyses of the Endo samples appear in Table II-5.

Included in Table II-6 are the analyses due to Leeder (1969) of cuticle, and of the tryptic residue and digest corresponding to the maximum dissolution (20%) he could obtain by tryptic digestion of cuticle. His cuticle was prepared by mechanical disruption of whole fibre in formic acid using a Vibromix agitator.

TABLE II-1
SPECIFICATION OF SAMPLES

Sample	Cuticle to pronase ratio	Time of treatment in days	Percent of whole cuticle represented by sample	Percent recovery of anhydroamino acids
Cuticle A	-	-	100	92.3
Cuticle B	-	-	100	93.7
Exo A	20 : 1	4	64	79.8
Exo B	20 : 1	7	64	72.1
Exo C	100 : 1	28	69	78.3
Endo A	20 : 1	4	36	33.8
Endo B	20 : 1	49	36	22.7
Endo C	100 : 1 then 50 : 1	14 14	*Between 31 & 36	30.1
Endo D	100 : 1	28	31	43.0
Performic Digest	-	1	63	71.9
Performic Residue	-	1	37	89.0

* Not measured experimentally

Reactions referred to were performed at 37°C

TABLE II-2

AMINO-ACID ANALYSES (MOLE %) OF CUTICLE

	<u>Cuticle A</u>	<u>Cuticle B</u>	<u>*Cuticle C</u>
CySO ₃ H	1.01	0.74	0.36
Asp	3.42	3.50	3.92
Thr	4.25	4.62	4.91
Ser	15.22	13.48	13.55
Glu	8.78	8.57	8.94
Pro	9.41	11.63	9.29
Gly	8.66	7.67	9.61
Ala	5.75	5.81	5.66
Val	7.57	7.45	6.59
½Cys	13.94	15.58	14.43
Met	0.30	0.37	0.38
Ileu	2.73	2.60	2.53
Leu	6.57	5.66	5.98
Tyr	2.96	2.70	2.92
Phe	1.80	1.58	1.86
Lys	2.80	2.67	2.88
His	0.79	0.83	0.96
Arg	4.04	4.54	4.79
Cit	-	-	0.48
CySO ₃ H+½Cys	14.95	16.32	14.79

*From King and Bradbury (1968)

TABLE II-3

AMINO-ACID ANALYSES (MOLE%) OF EXOCUTICLE

	<u>Exo A</u>	<u>Exo B</u>	<u>Exo C</u>	<u>Av. Exo</u>
CySO ₃ H	1.20	1.41	1.31	1.31
Asp	2.12	1.98	2.08	2.06
Thr	3.38	4.03	4.27	3.89
Ser	12.81	9.69	13.06	11.85
Glu	8.59	8.81	8.27	8.56
Pro	10.46	13.72	12.90	12.36
Gly	9.12	8.76	8.08	8.65
Ala	6.93	6.50	5.89	6.44
Val	8.51	8.31	7.65	8.16
½Cys	17.85	18.56	19.53	18.64
Met	0.17	0.19	0.12	0.16
Ileu	3.03	3.05	2.55	2.88
Leu	4.90	4.49	4.31	4.57
Tyr	2.20	1.75	1.92	1.96
Phe	1.20	1.13	1.16	1.16
Lys	2.33	2.04	1.81	2.06
His	0.58	0.54	0.44	0.52
Arg	4.62	5.04	4.65	4.77
CySO ₃ H+½Cys	19.05	19.97	20.84	19.95

TABLE II-4

AMINO-ACID ANALYSES (MOLE %) OF PRONASE, AND
ENDOCUTICLE CONTAMINATED WITH PRONASE

	<u>Pronase</u>	<u>Endo A</u>	<u>Endo B</u>	<u>Endo C</u>	<u>Endo D</u>
CySO ₃ H	0.00	0.70	0.72	0.69	1.19
Asp	12.27	7.91	8.40	7.64	7.21
Thr	9.27	5.96	5.81	5.55	6.05
Ser	7.50	9.87	11.33	10.72	9.94
Glu	5.35	9.79	9.88	10.20	9.69
Pro	3.35	7.91	8.92	8.97	8.37
Gly	14.47	8.56	8.74	8.39	8.97
Ala	11.31	6.96	7.03	6.90	7.21
Val	8.15	7.47	7.46	6.90	8.26
½Cys	1.18	2.26	2.29	2.81	1.04
Met	0.86	0.76	0.83	0.90	0.78
Ileu	3.59	4.09	3.63	3.71	4.22
Leu	5.49	9.01	8.45	8.52	9.98
Tyr	5.37	3.67	3.82	4.20	3.18
Phe	3.13	4.23	2.93	3.90	4.12
Lys	3.12	4.05	4.02	4.35	4.03
His	1.79	1.11	1.08	1.26	1.05
Arg	3.80	5.70	4.66	4.39	4.71
CySO ₃ H+½Cys	1.18	2.96	3.01	3.50	2.23

TABLE II-5

AMINO-ACID ANALYSES (MOLE %) OF ENDOCUTICLE
(corrected for pronase)

	<u>Endo A</u>	<u>Endo B</u>	<u>Endo C</u>	<u>Endo D</u>	<u>Av. Endo</u>
CySO ₃ H	0.80	0.82	0.73	1.23	0.90
Asp	7.34	7.88	7.38	7.05	7.41
Thr	5.52	5.35	5.33	5.96	5.54
Ser	10.01	11.87	10.91	10.02	10.69
Glu	10.44	10.51	10.49	9.84	10.31
Pro	8.42	9.55	9.25	8.50	8.92
Gly	7.77	7.97	8.04	8.81	8.15
Ala	6.39	6.47	6.64	7.08	6.65
Val	7.40	7.38	6.84	8.27	7.47
½Cys	2.42	2.44	2.90	1.03	2.20
Met	0.75	0.83	0.90	0.77	0.81
Ileu	4.17	3.64	3.72	4.23	3.94
Leu	9.54	8.86	8.71	10.12	9.31
Tyr	3.45	3.61	4.13	3.11	3.58
Phe	4.38	2.91	3.94	4.15	3.85
Lys	4.20	4.14	4.43	4.06	4.21
His	1.02	0.99	1.23	1.03	1.07
Arg	5.98	4.78	4.43	4.74	4.98
CySO ₃ H+½Cys	3.22	3.26	3.63	2.26	3.10

TABLE II-6

AMINO-ACID ANALYSES (MOLE %) OF CUTICLE, EXOCUTICLE,
ENDOCUTICLE, PERFORMIC DIGEST AND PERFORMIC RESIDUE

	<u>Av. Cuticle*</u>	<u>Av. Exo</u>	<u>Av. Endo</u>	<u>Performic Digest</u>	<u>Performic Residue</u>
CySO ₃ H	0.88	1.31	0.90	19.12	10.97
Asp	3.46	2.06	7.41	3.35	7.81
Thr	4.44	3.89	5.54	4.67	4.97
Ser	14.34	11.85	10.69	14.58	10.14
Glu	8.67	8.56	10.31	9.47	11.34
Pro	10.51	12.36	8.92	11.85	7.79
Gly	8.17	8.65	8.15	7.93	9.88
Ala	5.78	6.44	6.65	5.95	6.26
Val	7.51	8.16	7.47	7.68	6.77
½Cys	14.75	18.64	2.20	0.00	0.00
Met	0.34	0.16	0.81	0.00	0.00
Ileu	2.67	2.88	3.94	2.03	3.25
Leu	6.12	4.57	9.31	5.03	7.22
Tyr	2.83	1.96	3.58	0.15	0.28
Phe	1.69	1.16	3.85	1.29	2.26
Lys	2.74	2.06	4.21	1.66	5.37
His	0.81	0.52	1.07	0.71	1.39
Arg	4.29	4.77	4.98	4.53	4.30
CySO ₃ H+½Cys	15.63	19.95	3.10	19.12	10.97

*Average of Cuticle A and Cuticle B from Table II-2

TABLE II-7

AMINO-ACID ANALYSES (MOLE %) OF CUTICLE, EXOCUTICLE,
ENDOCUTICLE, TRYPTIC RESIDUE AND TRYPTIC DIGEST

	<u>Cuticle</u>	<u>*Cuticle</u>	<u>Av. Exo</u>	<u>Av. Endo</u>	<u>*Tryptic Residue</u>	<u>*Tryptic Digest</u>
Asp	3.46	3.58	2.06	7.41	3.24	15.13
Thr	4.44	5.05	3.89	5.54	4.98	4.65
Ser	14.34	14.82	11.85	10.69	17.05	7.78
Glu	8.67	9.35	8.56	10.31	9.36	12.63
Pro	10.51	4.75	12.36	8.92	4.40	3.92
Gly	8.17	10.91	8.65	8.15	10.31	15.68
Ala	5.78	5.66	6.44	6.65	5.98	6.36
Val	7.51	6.55	8.16	7.47	6.11	3.02
Met	0.34	0.41	0.16	0.81	0.33	1.52
Ileu	2.67	2.34	2.88	3.94	2.41	4.48
Leu	6.12	5.92	4.57	9.31	5.96	8.32
Tyr	2.83	3.04	1.96	3.58	3.27	4.03
Phe	1.69	1.92	1.16	3.85	1.97	2.07
Lys	2.74	2.39	2.06	4.21	2.50	4.94
His	0.81	1.11	0.52	1.07	0.99	0.87
Arg	4.29	4.97	4.77	4.98	3.81	4.42
Cit	-	0.89	-	-	1.25	0.28
CySO ₃ H+½Cys	15.63	15.75	19.95	3.10	16.05	0.00

*From Leeder (1969)

N.B. CySO₃H and ½Cys are not recorded separately in this Table

TABLE II-8

POLAR AND NON-POLAR RESIDUES, AND CROSS-LINKS (MOLE %) IN
WHOLE FIBRE, CUTICLE, EXOCUTICLE, AND ENDOCUTICLE

	<u>*Whole Fibre</u>	<u>Cuticle</u>	<u>Exocuticle</u>	<u>Endocuticle</u>
POLAR :	29.08	19.97	17.97	27.98
NON-POLAR :	70.92	80.03	82.03	72.02
CROSS-LINKS ($\text{CySO}_3\text{H} + \frac{1}{2}\text{Cys}$) :	9.99	15.63	19.95	3.10

*From Chapman and Bradbury (1968). CySO_3H (0.06%) is omitted
from cross-links for whole fibre.

TABLE II-9

POLAR RESIDUES (MOLE %) IN WHOLE FIBRE, CUTICLE, EXOCUTICLE,
AND ENDOCUTICLE

	<u>*Whole Fibre</u>	<u>Cuticle</u>	<u>Exocuticle</u>	<u>Endocuticle</u>
Asp	6.52	3.46	2.06	7.41
Glu	12.18	8.67	8.56	10.31
Lys	2.97	2.74	2.06	4.21
His	0.87	0.81	0.52	1.07
Arg	6.48	4.29	4.77	4.98

*From Chapman and Bradbury (1968).

N.B. The analysis did not distinguish between aspartic acid and
asparagine, or between glutamic acid and glutamine.

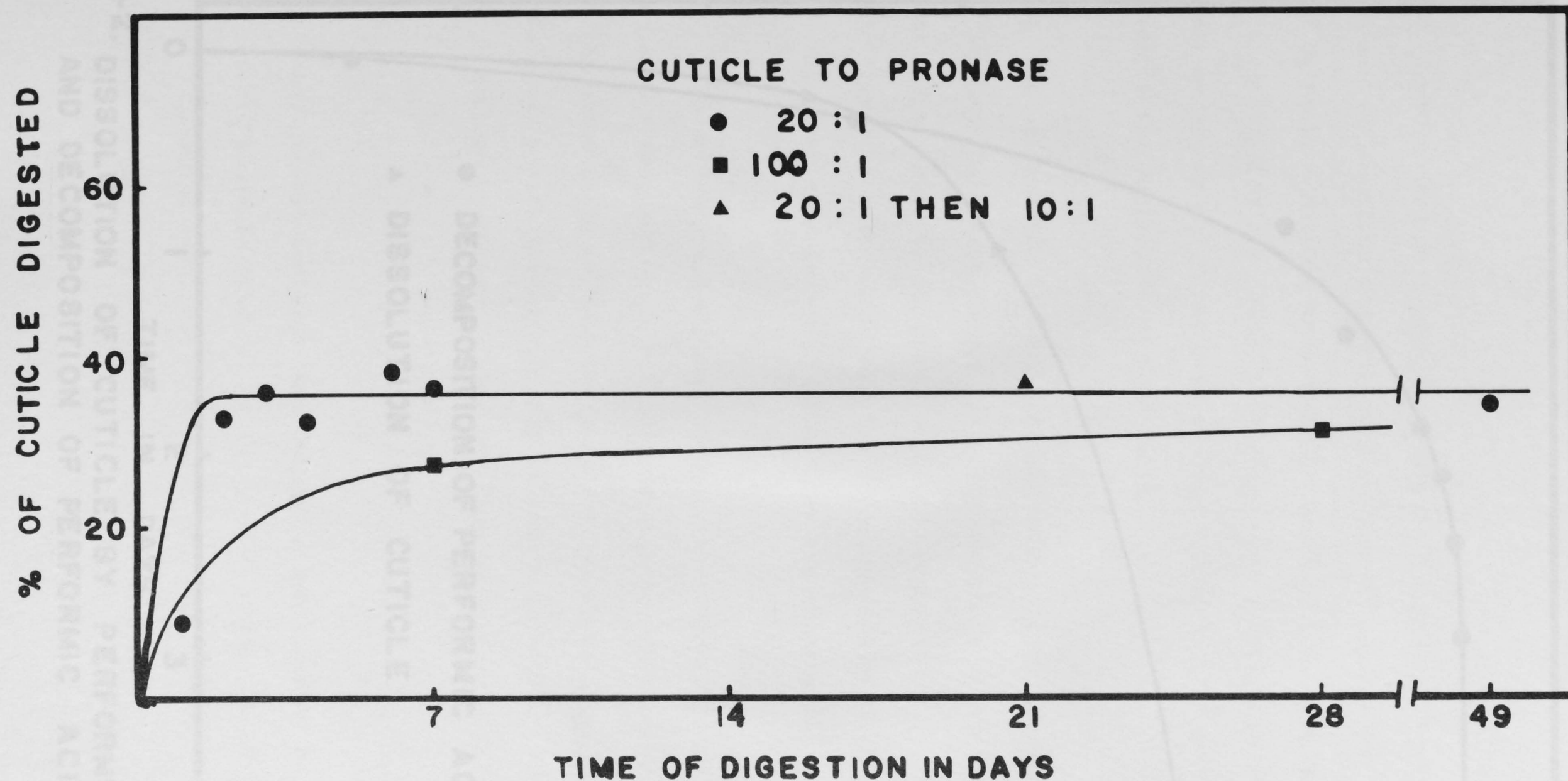


FIG. II-1. PRONASE DIGESTION AT 37° C OF CUTICLE

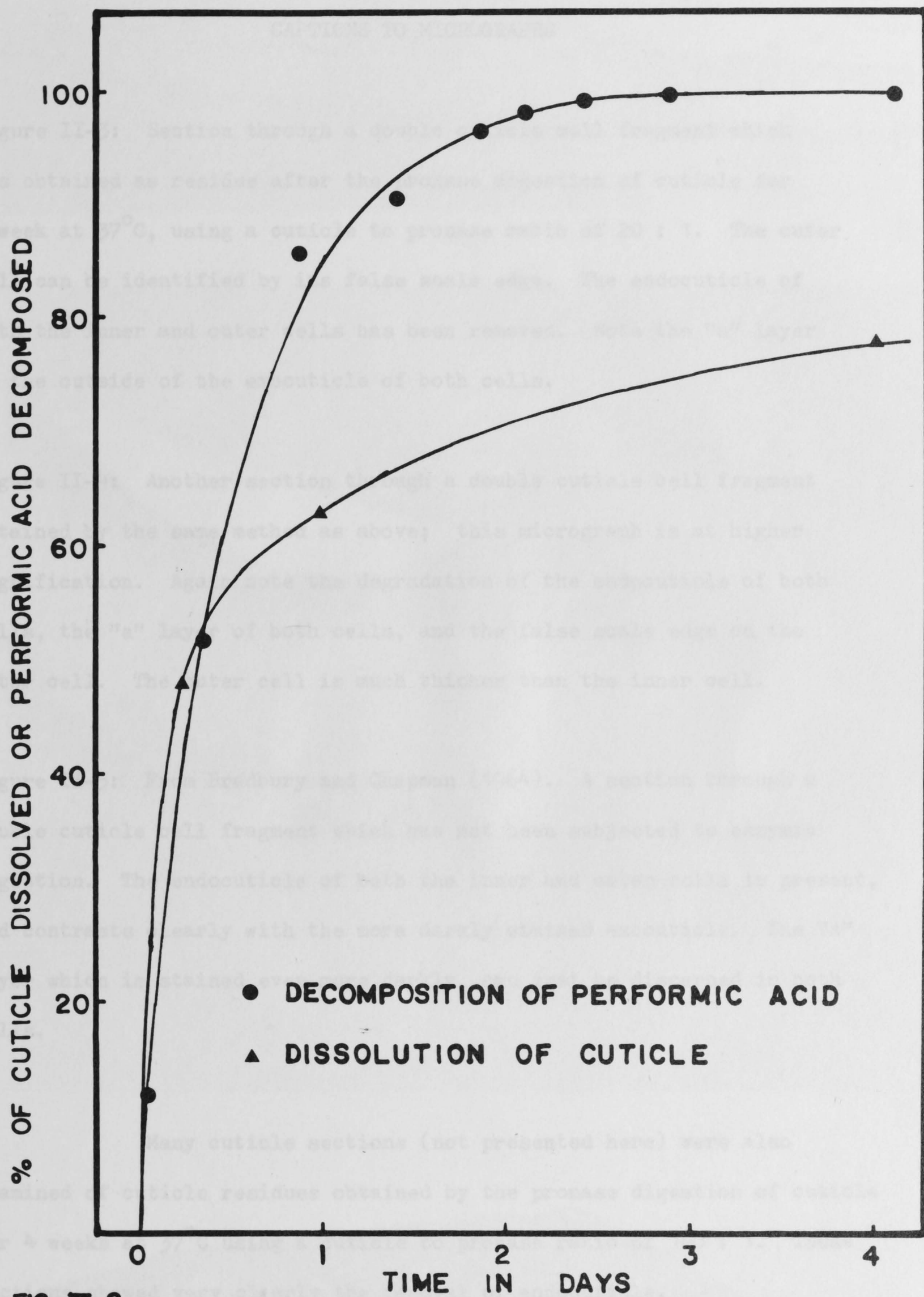


FIG. II-2. DISSOLUTION OF CUTICLE BY PERFORMIC ACID, AND DECOMPOSITION OF PERFORMIC ACID

CAPTIONS TO MICROGRAPHS

Figure II-3: Section through a double cuticle cell fragment which was obtained as residue after the pronase digestion of cuticle for 1 week at 37°C, using a cuticle to pronase ratio of 20 : 1. The outer cell can be identified by its false scale edge. The endocuticle of both the inner and outer cells has been removed. Note the "a" layer on the outside of the exocuticle of both cells. II-3

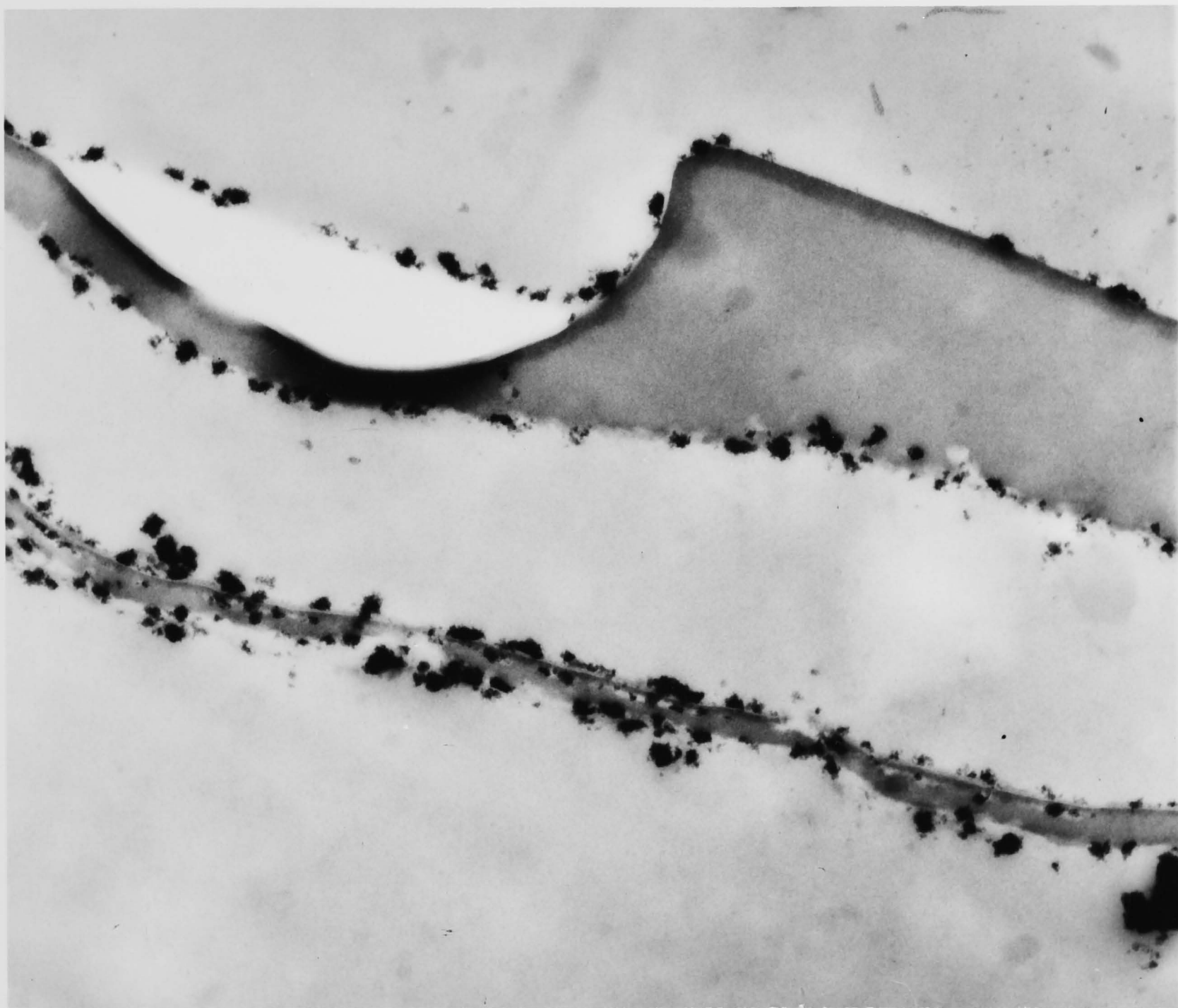
Figure II-4: Another section through a double cuticle cell fragment obtained by the same method as above; this micrograph is at higher magnification. Again note the degradation of the endocuticle of both cells, the "a" layer of both cells, and the false scale edge on the outer cell. The outer cell is much thicker than the inner cell.

Figure II-5: From Bradbury and Chapman (1964). A section through a double cuticle cell fragment which has not been subjected to enzymic digestion. The endocuticle of both the inner and outer cells is present, and contrasts clearly with the more darkly stained exocuticle. The "a" layer which is stained even more darkly, can just be discerned in both cells.

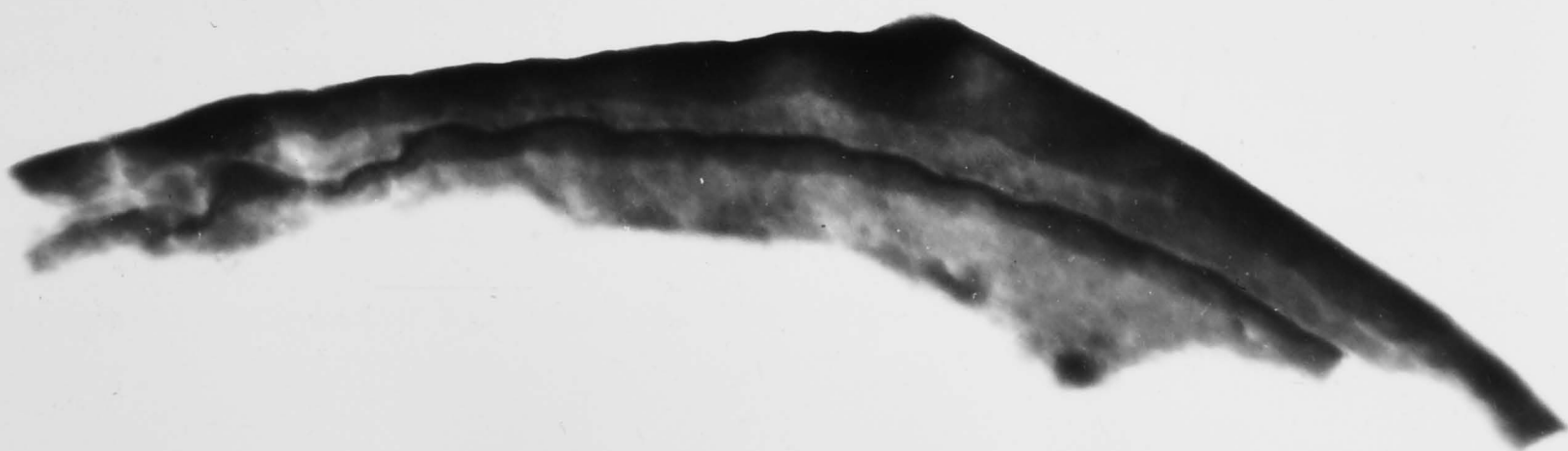
Many cuticle sections (not presented here) were also examined of cuticle residues obtained by the pronase digestion of cuticle for 4 weeks at 37°C using a cuticle to pronase ratio of 100 : 1. These sections showed very clearly the removal of endocuticle. II-4



II-3



II-4



II-5

4. Discussion

4(a). Digestion of cuticle by pronase as a function of time

We see at once from Figure II-1 that the cuticle is not completely dissolved away however long the digestion is continued. Instead the data is consistent with the exo/endo model, and the percentage of cuticle dissolved reaches a plateau, at about 36% if a cuticle to pronase ratio of 20 : 1 is used. This plateau is not due simply to the consumption by autolysis of the pronase available for reaction, since the percentage dissolved after 21 days had not increased significantly beyond 36% even though an additional amount of pronase had been added after the first eight days of the digestion.

Digestion with the lower concentration of enzyme is slower but approaches the plateau with time. By four weeks 31% of the cuticle has dissolved.

4(b). Electron microscopy of cuticle residue obtained by pronase digestion

The electron micrographs of sections of residues of double cuticle cell fragments seen in Figures II-3 and II-4, are notable for the extreme degradation shown of material underlying the exocuticle of both the inner and outer cuticle cell layers. In both figures the exocuticle of both the inner and outer cuticle cell layers has a clearly delineated "a" layer. Exocuticle appears intact. Each section is representative of the sample from which it came since very many other sections exhibiting the same degradation were observed in each sample. The electron micrograph due to Bradbury and Chapman (1964) in Figure II-5 shows an unmodified cuticle fragment having an inner and outer cuticle layer. The less heavily stained endocuticle of both layers is intact, and there is no empty space in the endocuticle region between the exocuticle regions of the inner and outer layers. Hence the results confirm that the material dissolved by pronase, approximately one-third by weight of the cuticle, is in fact the endocuticle.

4(c). Amino-acid analyses of cuticle

Except for cysteic acid the analyses of Cuticle A and Cuticle B in Table II-2 agree reasonably well with that of Cuticle C due to King and Bradbury (1968). Evidently the severe mechanical action of the Polytron (which was not used by King and Bradbury in preparing their sample) has caused oxidation of about 7% and 5% of the disulphide bonds of Cuticle A and Cuticle B respectively. However the corresponding decrease in cystine is not a significant decrease in the proportion of cystine, and so the amount of exocuticle made available to pronase digestion, by prior fission of some disulphides during the preparation of the cuticle, is not significant. That the composition of the cuticle preparation is reasonably consistent from batch to batch is shown in the comparison of the analyses for the two samples A and B, though the variation in cysteic acid must be noted. This suggests that the effect mentioned above due to the Polytron is not consistent in its severity. The other smaller differences reflect the reproducibility of the experimental technique.

4(d). Amino-acid analyses of exocuticle and endocuticle

The averaged analyses of exo- and endocuticle are compared in Table II-6. If we assume that cysteic acid is produced from cystine during preparation of the cuticle, then in order to compare the actual half cystine contents of exo- and endocuticle in the intact fibre, we must compare the sums of cysteic acid and half cystine in these two components.

By far the most striking feature of the analyses of exo- and endocuticle is the very high content of half cystine plus cysteic acid in the exocuticle, 19.95% or one disulphide bond in every five amino-acid residues, compared to the very low amount of 3.10% in the endocuticle. This vindicates the belief that resistance of exocuticle to digestion by pronase is due to the inaccessibility towards the enzyme of this highly cross-linked component.

The amount of half cystine in endocuticle, 2.20% or only one disulphide bond in fifty amino-acid residues, is not sufficient to prevent digestion. As the action of pronase is not specific for particular kinds of peptide bonds, the enzyme can digest away all the endocuticle, some of the peptide fragments released being cross-linked owing to the small amount of cystine present.

Exocuticle is significantly richer than endocuticle in cysteic acid plus cystine, and proline, in that order. Endocuticle is significantly richer than exocuticle in methionine, aspartic acid, phenylalanine, histidine, lysine, leucine, tyrosine, threonine, and isoleucine, in that order.

Table II-8 shows the distribution of polar and non-polar residues, and the content of cross-links, for exo- and endocuticle; the corresponding values for cuticle and whole fibre are included for comparison. Thus exocuticle is significantly less (by two-thirds) than endocuticle in polar residues, and is a little higher (14%) than endocuticle in non-polar residues. The polar residues account for just over one-quarter of the endocuticle, but they only account for about one-fifth to one-sixth of the exocuticle.

The content of the individual polar residues is shown in Table II-9 for whole fibre, cuticle, exocuticle, and endocuticle. Cuticle contains significantly less aspartic acid, glutamic acid, and arginine, than whole fibre. Exocuticle contains significantly less aspartic acid, lysine, and histidine, than cuticle. Exocuticle therefore is significantly less rich than the whole fibre in all the polar residues. Hence the lack of polarity in the cuticle compared to the whole fibre, previously noted by Bradbury, Chapman, and King (1965), is due to lack of polarity in the exocuticle.

Thus comparison of exocuticle, cuticle, and whole fibre, highlights the great lack of polar residues and the large extent of cross-linking in the exocuticle. Owing to these properties, the exocuticle must play an important role in the surface barrier to diffusion of dye and acid molecules

into wool (Millson and Turl, 1950; Lindberg, 1953; Medley and Andrews, 1959). The lack of polarity of the exocuticle supports the postulate of Lindberg (1953) that the surface barrier to diffusion of such molecules into the fibre is electrical in nature.

Since the endocuticle contains the degenerated nucleus and cytoplasmic material forced to the endo side by the developing keratin (e.g. Birbeck and Mercer, 1957), there must be some non-protein, phosphates for example, in the endocuticle. Now from the analyses of cuticle, exocuticle, and endocuticle, one can calculate the proportion of exo protein to endo protein in cuticle protein. If both the exo- and endocuticle are pure protein, the proportion calculated should equal the proportion of exo- to endocuticle obtained from the data for digestion by pronase. If however endocuticle contains some non-protein, the proportions obtained by the two methods should differ, and the extent of the difference would indicate the amount of non-protein in endocuticle.

Theoretically, for all the amino-acids the equation $\% \text{ in Cuticle} = (\% \text{ in Exocuticle})x + (\% \text{ in Endocuticle})(1-x)$ will yield the same value of x , the fraction of exo protein in cuticle protein. In practice, the values of x obtained will vary somewhat, but the most reliable values can be had by solving the equation for those amino-acids showing the greatest difference between exo - and endocuticle.

For each amino-acid its mole percent in cuticle should lie between the values for exo- and endocuticle. But such is not the case for serine, alanine, isoleucine, and arginine, and so the equation in x does not hold for these residues.

The equation was solved for x for all the amino-acids below, each of which shows a substantial difference between exo- and endocuticle.

Residue	Tyr	Pro	His	Thr	Leu	Lys
100x (%)	46	46	47	67	67	68

Residue	Met	Asp	CySO ₃ H+½Cys	Phe
100x (%)	72	74	74	80

Av. 100x (%) : 64 (72 if exclude Tyr, Pro and His)

The average value of x , expressed as a percentage, is 64%. Remarkably, this is identical with the percentage by weight of exocuticle in cuticle, obtained from digestion with pronase, and so implies that neither exo- nor endocuticle contain non-protein. Unfortunately however, the values of x for tyrosine, proline, and histidine, stand as a group well below the values for the remaining residues. If we exclude these three, the average percentage obtained for exo protein in cuticle protein is 72%. Thus the percentage of endo protein in cuticle protein, 28%, contrasts with the percentage by weight of endocuticle in cuticle, 36%. From this one can calculate that there is 31% by weight of non-protein in endocuticle, a considerable amount.

On the other hand it may be questionable to exclude tyrosine, proline, and histidine from the averaging calculation, since for these residues the differences between exo- and endocuticle are not insignificant, and expressed as a percentage of the higher value in each case, are 45%, 28%, and 52% respectively.

Finally we recall that technical difficulties made it impossible to obtain accurate estimates of the recovery of anhydroamino-acids for the endo samples, and so we cannot obtain the precise proportion of non-protein in endocuticle by this method either.

In contrast to pronase, trypsin is specific in its action and attacks amide bonds derived from the carboxyl groups of lysine and arginine (Fieser and Fieser, 1961) and so the peptide fragments which can be released by trypsin are much longer than those released by pronase. Thus tryptic attack on cuticle is limited by the specificity of the enzyme as well as by the cross-links of the exocuticle, and Leeder (1969) could only obtain a maximum dissolution of 20% with trypsin. There was no cystine in his tryptic digest (Table II-7) and no doubt the already long peptide fragments which also happened to be cross-linked by endocuticle cystine, were so large that they could not be disentangled from the residue.

In Table II-7 are compared pronase residue (exocuticle) with tryptic residue, and also pronase digest (endocuticle) with tryptic digest. The analysis of Leeder's cuticle preparation is presented for comparison. Proline is unaccountably low in his cuticle. Since only one-fifth of the cuticle dissolved with trypsin and one-third dissolved with pronase, one cannot expect much correlation between the pronase and trypsin results. Yet surprisingly, many of the amino-acids which exhibit a significant difference for exo- and endocuticle, also exhibit a significant difference in the same direction and often of a similar magnitude, for the tryptic residue and digest. On the other hand if one compares actual analyses rather than differences in analyses, the discrepancies are often considerable. Thus for some amino-acids, exocuticle is very different to tryptic residue, and endocuticle is very different to tryptic digest.

The conclusion from the data is that tryptic digestion has effected a partial separation of cuticle into exo- and endocuticle. The exocuticle has not been extracted. Only part of the endocuticle has been extracted and this material has a somewhat different composition to endocuticle owing to the selective action of trypsin.

4(e). Dissolution of cuticle with performic acid

The amount of cuticle dissolved by performic acid at 37°C clearly levels off with time, as seen for the few samples in Figure II-2. However the stability of the performic acid was in question and so its decomposition with time was then studied. Comparison of the two curves in Figure II-2 indicates that the levelling off in cuticle dissolution is due to the rapid decomposition of the performic acid. By one day 63% by weight of the cuticle has dissolved and cystine is no longer present in either the residue or digest (Table II-6). From this time onward both the dissolution of cuticle and the decomposition of performic acid begin to slow down markedly and this shows the controlling influence of the latter effect upon the former.

It is probable that after oxidation of disulphide bonds the exocuticle swells greatly in comparison to the endocuticle, owing to the repulsion between the numerous sulphonic acid groups. Proteins are then preferentially disentangled from the exocuticle. No doubt the formic acid present helps swell both the exo- and endocuticle, and with time, material dissolves from the endocuticle also, possibly until the whole of the cuticle dissolves. The proportion of cuticle extracted after one day is almost identical with the proportion of cuticle obtained as residue after digestion with promase. This suggests that the exocuticle may be extracted after one day, and so we compare the analyses of exo- and endocuticle with those of the performic digest and residue obtained after this time (Table II-6).

4(f). Amino-acid analyses of performic digest and residue

The agreement between the two sets of analyses is notable and is much better than for the comparison of tryptic residue and digest with exo- and endocuticle. Due to the similarities between exocuticle and performic digest, and between endocuticle and performic residue, those amino-acids showing significant differences between exo- and endocuticle also show significant differences between performic digest and residue. Note especially aspartic acid, proline, isoleucine, leucine, tyrosine, phenylalanine, lysine, and histidine. In the performic digest and residue cystine has of course been oxidised to cysteic acid, and the zero methionine values are due to conversion to methionine sulphone. Tyrosine is much lower in the performic digest and residue than in the exo- and endocuticle respectively, probably because of decomposition by oxidation. Note that for leucine, there is significant disagreement of the performic digest and residue with exo- and endocuticle. The above similarities notwithstanding, there is one particularly sharp disagreement: between endocuticle and performic residue for cysteic acid plus half cystine. This proves that

performic acid has not cleanly separated exo- and endocuticle, for endocuticle contains only about 3% of these two residues. As we have seen, increasing amounts of endocuticle are dissolved after longer and longer times, and so it is reasonable to expect some dissolution of this component after one day only.

Because of the similarities noted above, the choice of one day as the time of treatment to produce the samples for analysis, was rather fortuitous. Though only a partial separation was effected, the performic acid was preferential in its attack on exocuticle. Its action thus resembles that of the peracetic oxidation followed by ammoniacal or alkaline extraction tried by earlier workers. The results obtained with performic acid are confirmatory evidence for the separation obtained with pronase.

Returning to the work with pronase, we must conclude that the results of the dissolution experiments, electron microscopy, and amino-acid analysis, constitute reasonable proof of the separation of exocuticle and endocuticle with this enzyme. Thus the analyses here reported are the first analyses achieved of exocuticle and endocuticle.

C H A P T E R I I I

BILATERAL DIFFERENTIATION IN FINE WOOL FIBRE

1. Introductory Review

1(a). Histological differences, seen in the electron microscope, only between orthocortex and paracortex.

Bilateral differentiation within the cortex is clearly revealed in appropriately stained cross-sections of fine wool fibres viewed at low magnification in the electron microscope (e.g. Rogers, 1959b).

Roughly 60% of the area of a cross-section is occupied by the orthocortex, and the difference observed at low magnification between the two segments is chiefly due to the orthocortical cells exhibiting a noticeably different internal arrangement within their own cross-sections compared to the arrangement within the paracortical cells.

In orthocortical cells, macrofibrils are clearly delineated by the material of the inter-macrofibrillar boundaries, which are not easily distinguishable from the cell membrane complex separating orthocortical cells from each other. Paracortical cells on the other hand show no delineation of macrofibrils, and in consequence the boundaries between individual paracortical cells are clearly visible.

Large dark nuclear remnants are visible within most of the paracortical cells, whereas in orthocortical cells the nuclear remnants are often difficult to distinguish, and apparently are smaller in size and occur less frequently than their counterparts in paracortical cells.

An electron micrograph, due to Dobb et al. (1961), of a longitudinal section of a Merino fibre shows larger nuclear remnants in the paracortex.

At higher magnification in the electron microscope, individual macrofibrils of the paracortex of Merino fibre can be distinguished, since both inter-macrofibrillar material and flaws in the microfibril/matrix structure can be seen defining the boundaries of the macrofibrils (Rogers, 1959b). The difficulty of distinguishing the macrofibrils of the paracortex at low magnification, is probably due to the presence of only these ill-defined flaws along most of the boundary separating macrofibrils. In the paracortex, inter-macrofibrillar material accumulates most in the spaces where several of the approximately round macrofibrils abut, rather than along the boundary between two macrofibrils only.

Furthermore, at the higher magnification, the arrangement of the microfibrils in the microfibril/matrix structure of the orthocortex is often seen to be different to the corresponding arrangement in the paracortex (Rogers, 1959b; Fraser, MacRae, and Rogers, 1960). It is usual for the microfibrils of the orthocortex to be arranged in whorls. This arrangement is not common in the paracortex, where almost regular hexagonal packing of much more clearly defined microfibrils is often found.

1(b). Amino-acid analyses of orthocortex and paracortex.

A reliable separation procedure for obtaining ortho- and paracortical cells has been developed recently (Bradbury, Chapman, and King, 1967; Chapman, 1967; Chapman and Bradbury, 1968). Since unlike earlier separation procedures which we will later discuss, this method causes barely any chemical degradation, and so we here describe the protein chemistry of ortho- and paracortex prepared by this method.

Briefly, the separation is achieved by preferential deposition of gold in the paracortex, then disruption of the fibre by ultrasonication in formic acid and separation of the more heavily stained paracortical cells from the orthocortical cells by centrifugation in a density gradient. The only modification of amino-acids occurring during the preparation is

the oxidation of some cystine to cysteic acid, and of some methionine to methionine sulphone. Since their amino-acid analyses of virgin wool and wool stained bilaterally with gold showed complete agreement, except in the matter of oxidation of cystine and methionine in the latter sample, these workers concluded that in gold stained ortho- and paracortical cells this oxidation was again the only modification that occurred.

They found that orthocortical cells contain significantly more tyrosine (26%), glycine (16%), leucine (12%), and phenylalanine (12%), and less half-cystine plus cysteic acid (14%), and histidine (14%) than paracortical cells. It is notable that these differences, though significant, are nevertheless fairly small, and that none of the acidic or basic amino-acids (except histidine) shows significant differences.

Chapman (1967) has reported amino-acid analyses of the SCMKA (low sulphur) and SCMKB (high-sulphur) protein fractions of both ortho- and paracortical cells, and mention is made of these analyses by Bradbury, Chapman, and King (1967). After correcting for the amount of gold in his samples, Chapman found that for orthocortical cells these low- and high-sulphur fractions accounted for all but 7% of the total protein, and that for paracortical cells they accounted for all but 6% of the total protein. The ratio of the yields of SCMKA to the yield of SCMKB was 0.82 : 1 for orthocortical cells, and 0.71 : 1 for paracortical cells. Unfortunately it is probable that Chapman did not achieve a complete separation of low- and high-sulphur fractions since his SCMKB samples contain some methionine which is normally absent from SCMKB (Gillespie and Inglis, 1965). It is likely that this incomplete separation was due to the difficulty of reducing all the disulphides of the gold stained ortho- and paracortical cells to thiols, since some of the disulphides had already been oxidised to sulphonic acids by Au(III). Fractionation into alpha- and gamma-keratins would have circumvented this problem.

Very recently, amino-acid analyses of low- and high-sulphur proteins of both ortho- and paracortical cells have also been reported by Kulkarni, Robson, and Robson (1971). The method they used to separate ortho- and paracortical cells is mildly degradative, but nevertheless stands with the method of Bradbury, Chapman, and King (1967) in being much less degradative than the severe separation procedures developed by earlier workers. The separation of Kulkarni et al. involved tryptic digestion of wool, followed by ultrasonic disintegration, after which the mixture of liberated cortical cells was separated into light and heavy fractions by centrifugation in a mixture of carbon tetrachloride and xylene. Electron microscopy was used to confirm that the light and heavy cells were ortho- and paracortical cells respectively. The separation left some residual paracortex which was not separated into individual paracortical cells. The paracortical analyses reported are the average of the analyses for paracortical cells and the residual paracortex.

Comparison of analyses due to Kulkarni and coworkers with those of Bradbury and coworkers, for both ortho- and paracortical cells, shows reasonable agreement between the two sets of analyses, though the following exception is noteworthy. There is a large discrepancy for the sum of half-cystine and cysteic acid. Kulkarni et al. and Bradbury et al. report 12.1% and 7.5% respectively for orthocortical cells, and 15.2% and 8.7% respectively for paracortical cells (values in mole %). The analyses of both groups of workers show that for acidic residues (excluding cysteic acid) and for basic residues, there is no significant difference between ortho- and paracortical cells.

Kulkarni et al. obtained low- and high sulphur fractions in the form of alpha- and gamma-keratoses for both ortho- and paracortex. For orthocortical cells the alpha- and gamma-keratoses comprised almost the entire protein, since these workers obtained a yield of only 1% for the residual beta-keratose; they found that the ratio of the yield of

alpha-keratose (low-sulphur protein) to the yield of gamma-keratose (high-sulphur protein) was 1.49 : 1. For paracortical cells the residue of beta-keratose amounted to 15% of the protein, and a ratio of 0.89 : 1 was found for the yield of alpha-keratose to the yield of gamma-keratose. Of the residual paracortex remaining after separation of ortho- and paracortical cells, 5% was obtained as beta-keratose, and a ratio of 1.11 : 1 was found for the yield of alpha-keratose to the yield of gamma-keratose. For paracortex the analyses reported for the alpha- and gamma-keratoses are again the mean of the corresponding analyses for the paracortical cells and the residual paracortex.

For both the alpha- and gamma-keratoses, Kulkarni et al. compare the analyses for the keratoses of ortho- and paracortex by dividing amino-acids into categories representing a "high-sulphur index" and an "alpha-helix index". The legitimacy of this practice is established by the following observations. In a number of low-sulphur fractions from wool, the proportion of alpha-helix increases with increasing amounts of glutamic acid, aspartic acid, leucine, lysine, and alanine (Crewther and Harrap, 1967; Corfield, Fletcher, and Robson, 1968), and since high-sulphur protein fractions from wool contain hardly any alpha-helix, the sum of the above amino-acids not only represents a crude "alpha-helix index" but is also a measure of the low-sulphur protein content. Furthermore, since numerous analyses have shown that three out of five residues in high-sulphur protein fractions are accounted for by cystine, serine, proline, and threonine, the sum of these amino-acids can be used as a crude "high-sulphur index" to indicate the amount of high-sulphur protein in a sample.

The results of Kulkarni et al. show that the high-sulphur index of the high-sulphur protein fraction of the paracortex is significantly greater than the high-sulphur index of the high-sulphur protein fraction of the orthocortex. They also show that the alpha-helix index of the low-sulphur fraction of the orthocortex is greater than the alpha-helix index of the low-sulphur fraction of the paracortex. As the results reveal

the paracortex to be richer than the orthocortex in both high-sulphur proteins and disulphide cross-links, Kulkarni et al. concluded that the paracortex was probably more densely and extensively cross-linked than the orthocortex.

1(c). Ratio of microfibrils to matrix.

If microfibrils can be equated with the low-sulphur proteins of wool, and matrix with the high-sulphur proteins (an hypothesis of some controversy: Crewther, Fraser, Lennox, and Lindley, 1965), then for both ortho- and paracortex, estimates of the ratio of low-sulphur to high-sulphur protein may be compared with estimates of the ratio of microfibrils to matrix. Such a comparison is made in Table III-1.

TABLE III-1.

RATIO OF MICROFIBRILS (%) TO MATRIX (%)

	Orthocortex	Paracortex
Leach et al. (1964)	80 : 20	50 : 50
Dobb (1970)	68 : 32	40 : 60
Chapman (1967)	45 : 55	41 : 59
Kulkarni et al. (1971)	60 : 40	50 : 50

The values calculated from Chapman, and from Kulkarni et al. are ratios of low-sulphur to high-sulphur protein. For Kulkarni et al. the ratio given for paracortex has been obtained by averaging the ratios calculated for paracortical cells and residual paracortex. The ratios cited for Leach et al. and Dobb, are direct estimates of microfibrils to matrix, not low-sulphur to high-sulphur. These two groups of workers have calculated their ratios on a volume, not a weight basis, unlike the ratios of Chapman, and Kulkarni et al.; hopefully any difference in density between microfibrils and matrix is sufficiently small to permit comparison of ratios obtained by the two methods. The ratios of Leach et al.

were obtained by measurements on electron micrographs; infiltration into the structure by the plastic embedding material and by the metal stain used could possibly distort the microfibril/matrix structure from its true dimensions. The ratios cited for Dobb are the averages of the range of values he reported; his estimates were based on electron diffraction studies.

The discrepant ratios due to Chapman reflect the contamination of his high-sulphur samples with low-sulphur protein, a situation also indicated by the presence of methionine in his SCMKB. Thus if corrected, his actual values for microfibrils and matrix would increase and decrease respectively, and the resultant ratios would approach the other ratios in the Table.

It is significant that the ratios calculated on a weight basis are similar to those calculated on a volume basis, and this indicates that the density difference between microfibrils and matrix is not very large, and that distortions causing errors of measurement are not very large.

Also, the estimates of microfibrils to matrix are similar to the estimates of low-sulphur to high-sulphur proteins. This supports the correspondence of microfibrils with low-sulphur proteins, and matrix with high-sulphur proteins.

1(d). Treatments which show up the bilateral differentiation of the fibre.

We merely list these treatments here, but will interpret their significance in Section 1(f).

Prior to the discovery of the bilateral effect in 1953 by Horio and Kondo, a number of early workers reported results which, though attributed either to variability or freak damage of the fibre, were in fact dependent on the bilateral nature of the fibre. Thus for the following workers the fibre cortex showed a differential response under the conditions listed: alkaline and other chemical treatments (McMurtrie, 1886; von Bergen, 1929 and 1935; Hirabayashi, 1938; Freney, 1947; Lindley, 1947; Zahn and

Haselman, 1950; Goldsworthy and Lang, 1953); birefringence studies (Ohara, 1938 and 1939); variation of humidity (Woods, 1935); asymmetric keratinisation (Rudall, 1936); fungal attack (Race, 1946); deposition within the fibre of metal sulphides (Schoberl, 1942); and staining with organic dyes (Ohara, 1938 and 1939; Royer and Millson, 1940; Watkins, Royer, and Millson, 1944).

At last Horio and Kondo (1953) demonstrated the presence within the cortex, of two segments in the form of hemicylinders wound helically around each other in phase with the fibre crimp, so that one segment (the orthocortex) lies on the outside, and the other segment (the paracortex) lies on the inside of the curve of the crimp wave. They found that basic dyes, used on both whole fibres and cross-sections of fibres, stained the orthocortex. Strong alkali was shown to cause preferential swelling and loss in birefringence in the orthocortex.

The existence of bilateral differentiation was shortly confirmed by Mercer (1953), who obtained digestion of the orthocortex but not the paracortex with trypsin, in wool previously supercontracted in water at 120°C. The residual material retained its birefringence and so was identified as paracortex.

Following the lead given by Horio and Kondo, numerous workers produced bilateral staining in fibres with a wide range of organic dyes; the extensive literature of this research has been reviewed by Chapman (1967). Horio and Kondo had themselves produced staining on the inside of the crimp wave, apparently in the paracortex, but later workers thought that reversal of crimp had occurred under the particular dyeing conditions used, and that in fact the orthocortex had stained. In general, organic dyes have been shown to stain the orthocortex. The conclusion was reached by Horio, Kondo, Sekimoto, and Teramoto (1960) that basic dyes stain the orthocortex, and that with acid dyes, those containing only

sulphonic groups stain the paracortex, whereas those acid dyes containing both basic and sulphonic acid groups stain the orthocortex. Oxidative pretreatments of the fibre with performic acid, peracetic acid, and bromine have been used by some workers to produce staining of the paracortex by dyes which would normally have stained the orthocortex. Conversion of cystine to S-carboxymethyl groups enabled Kassenbeck (1967) to stain the paracortex preferentially with methylene blue.

1968; Bilateral deposition of metals in fibres has been studied by light and electron microscopy. Following deposition of metal sulphides in wool, using metallic salt solutions, by Elod, Nowotny, and Zahn (1940) and Schoberl (1942) (the latter of whom suspected that the deposits might have been asymmetrically distributed within the fibre), darker staining was achieved in the paracortex by Mercer, Golden, and Jeffries (1954), Thorsen (1958), and Priestley (1966), who all used sodium plumbite solution to stain the fibre a deep brown, presumably with lead sulphide. The formation of deposits of lead sulphide on refluxing wool with lead salts had enabled Smith and Harris (1936) to gauge the extent of breakage of disulphide bonds in oxidised wool, and so Ross (1955) used lead acetate over a range of pH in an attempt to obtain information concerning the distribution of sulphur in wool. At pH 7.0 black streaks of lead sulphide were produced in the paracortex of Merino fibre, apparently in the nuclear remnants.

post-stain Stannous chloride was long ago used by Becke (1919) and Daneel (1936) to stain wool; the latter worker observed dense streaky aggregates within the fibre. Ross (1955) therefore used this reagent in his investigation of bilateral differentiation. He found some evidence of light brown colouration in the orthocortex of Merino fibres. In stained fibres having radial rather than bilateral asymmetry, well defined cores were observed; in the nuclear remnants of the cortical cells within the core of each fibre, black crystallites of stannous sulphide were seen.

Merino fibres swollen in 98%-100% formic acid, then stained in gold chloride, and finally immersed in 25% formic acid, were found to have coarse dark crystallites of gold in the nuclear remnants within the paracortex and a background red colour distributed throughout the cortex as a whole (Laxer and Ross, 1954; Ross, 1955). This effect has lately been the subject of chemical and electron microscopic investigation (Bradbury, Chapman, and King, 1967; Chapman, 1967; Chapman and Bradbury, 1968; Bradbury, Ley, and Peters, 1971). Gold chloride staining of the paracortex of Merino fibres without any accompanying formic acid treatment has been studied with the electron microscope by Kulkarni, Robson, and Robson, (1971).

An apparently histochemical staining of the orthocortex has been obtained by treating wool with chromic, cobaltous, cupric, ferric, manganous, and nickelous ions, all in acidic nitrite solution (Mercer, 1954c; Thorsen, 1958; Corbett and Yu, 1964; Lang and Campbell, 1966; Priestley, 1966).

Mercuric carroting liquids have been found to stain the orthocortex (Mercer, 1954c) and staining of the paracortex of cross-sections of wool fibres was accomplished by exposing the sections to mercury vapour (Menkart and Coe, 1958). The electron microscope shows preferential staining of the paracortex, accompanied by deposition of submicroscopic precipitates and strong degradation of the structure in cross-sections of Merino fibres post-stained with ammoniacal mercury solutions (Kassenbeck, 1955b; Kassenbeck, 1967).

Precipitation of thallium sulphide and breakage of disulphide bonds occur when the paracortex is stained by post-staining cross-sections of bilateral fibres with alkaline thallium carbonate. Since both the precipitates and the chemical degradation obscure fine detail, this staining method was found unsuitable for electron microscopic investigations.

Similar reasons render unprofitable the inspection in the electron microscope of sections of fibres post-stained with ammoniacal solutions of cadmium, copper, and mercury (Derminot, Tashdomme, and Parisot, 1965; Kassenbeck, Jacquemart, and Monrocq, 1965; Kassenbeck, 1967).

Prior to sectioning, reduction with thioglycollic acid followed by staining with osmium tetroxide, has been used to produce good bilateral differentiation in the background histochemical staining seen in electron micrographs of fine wool fibres (Rogers, 1959 a & b; Filshie and Rogers, 1961; Leach, Rogers, and Filshie, 1964). Post-staining of these sections with lead salts or lead hydroxide improves the contrast between light and dark areas in the micrographs (Watson, 1958; Filshie and Rogers, 1961).

A deeper histochemical staining in the paracortex was visible in electron micrographs of fibres stained with silver nitrate or silver acetate (Derminot, Tasdhomme, and Parisot, 1965; Kassenbeck, 1965 a & b; Kassenbeck and Hagege, 1965); formation of appreciable amounts of lanthionine was found to occur during the staining, (Derminot, Tasdhomme, and Parisot, 1965; Kassenbeck, 1967). Electron microscopy showed that silver nitrate treatment, of wool reduced but not coupled, also gave darker histochemical staining in the paracortex (Nott and Sikorski, 1965); ammoniacal silver nitrate was shown to stain the paracortex in preference to the orthocortex, and the matrix in preference to the microfibrils, in both wool and S-carboxymethylated wool (Kassenbeck, 1967).

The electron microscopy of Kassenbeck (1967) showed that uranyl acetate, known to be specific for carboxyl groups and to behave like a basic dye (Kuhn, Grassman, and Hofman, 1959; Kassenbeck and Hagege, 1965; Huxley and Zusay, 1960), when used on untreated wool, stained the orthocortex in preference to the paracortex and the microfibrils in preference to the matrix. If the disulphide bonds were first converted to S-carboxymethyl groups however, the paracortex and microfibrils stained in preference to the orthocortex and matrix respectively. Kassenbeck used

lead hydroxide (Karnovsky, 1961) or lead citrate (Reynolds, 1963) to enhance considerably whatever contrast he obtained with uranyl acetate.

A marked preferential staining of the orthocortex has been produced in electron micrographs of fibre cross-sections post-stained with phosphomolybdic acid (Kassenbeck and Hagege, 1965) and phosphotungstic acid (Kassenbeck and Hagege, 1965; Bones and Sikorski, 1967; Kulkarni, Robson, and Robson, 1971), but no contrast could be obtained between microfibrils and matrix with these reagents (Kassenbeck, 1967).

Chapman (1967) has listed the details of those chemical and other treatments by which the reactivities of the ortho- and paracortex may be distinguished, and so now we merely group these treatments into categories.

Almost invariably, the essential feature here is the resistance offered by the paracortex under conditions which affect the orthocortex. (It is usual for the cuticle sheath to remain unaffected also).

Acid hydrolysis with hydrochloric or sulphuric acids dissolves orthocortex faster than paracortex; if the conditions are carefully controlled, the paracortex can be obtained as a residue free of orthocortex. Separated orthocortical cells dissolve faster than separated paracortical cells, when treated with hydrochloric acid (Elliott and Roberts, 1956; Elliott, Asquith and Rawson, 1959; Leveau, 1959a; Leach, Rogers and Filshie, 1964).

This last group of workers examined by electron microscopy cross-sections of Merino fibres treated for increasing times with boiling hydrochloric acid at pH 2 - a procedure which causes preferential release of aspartic acid from the protein. After 96 hours all the orthocortex was extracted, leaving a seemingly unaltered paracortex in which the original microfibril/matrix structure appeared intact. At times shorter than 96 hours, severe degradation in the orthocortex of inter-macrofibrillar material and of the interior of macrofibrils was observed. The treatment also removed

the cell membrane complex and the nuclear remnants of both segments.

However, Kulkarni, Robson, and Robson (1971) have lately shown that this same treatment releases aspartic acid from separated paracortical as well as from separated orthocortical cells, though the rate of release from paracortical cells is slower. Continuation of the hydrolysis to the stage where Leach et al. found complete dissolution of orthocortex, releases 26% of the total aspartic acid content of paracortical cells from those cells, and so the conditions used by Leach et al. did not leave the paracortex completely unchanged.

Prior treatment of wool in boiling sulphuric acid facilitates subsequent alkaline extraction which occurs in two stages: the orthocortex dissolves faster than the paracortex (Dusenbury, Mercer, and Wakelin, 1954; Dusenbury and Jeffries, 1955; Dusenbury and Menkart, 1955).

Alkali can cause swelling (or complete dissolution) and loss of birefringence of the orthocortex, and the preferential swelling of the orthocortex causes fibres to coil (Frenay, 1947; Goldsworthy and Lang, 1953; Horio and Kondo, 1953; Fraser and Rogers, 1954; Dusenbury and Coe, 1955; Dusenbury, and Jeffries, 1955; Fraser and Rogers, 1955a; Elliott and Roberts, 1956; Leveau, 1956b; Haly, 1957; Elliott, Asquith, and Rawson, 1959; Leveau, 1959a; Daveloose, Mazinque, and van Overbeke, 1960; Louw, 1960).

Dissolution of the orthocortex also results from treatment of the fibre with urea/bisulphite (Mercer, 1954d; Dusenbury, 1960), cupriethylenediamine (Leveau, 1959 a & b; Daveloose, Mazinque, and van Overbeke, 1960), formamide (Leveau, 1959a), and sodium sulphide solution (Daveloose, Mazinque, and van Overbeke, 1960).

Not only alkali, but also cadmium triethylenediamine (Suarez, 1966), glacial acetic acid, saturated urea solution (Fraser and Rogers, 1955a), and urea/bisulphite (Mercer, 1954d; Satlow and Kessler, 1958), destroy birefringence in the orthocortex.

Preferential supercontraction of the orthocortex has been effected by lithium bromide (Haly, 1957; Haly and Griffith, 1958), urea/bisulphite (Mercer, 1954d), and by heating in water (at 120°C), formamide, and aqueous phenol (Mercer, 1953). With lithium bromide, Banbaji (1970) has recently produced a greater supercontraction in separated orthocortical cells than in separated paracortical cells.

Peracetic acid oxidation followed by ammonia extraction swells the orthocortex (Mercer, 1953; Fraser and Rogers, 1955a), but if the oxidation is followed by immersion in water, the paracortex swells (Menkart and Coe, 1958).

Alkaline thioglycollate preferentially degrades and can even extract the orthocortex (Fraser and Rogers, 1953, 1955 c & d).

Remarkably, preferential attack and dissolution of the paracortex, not the orthocortex, can be achieved with bromine (Leveau, 1959a; Menkart and Coe, 1958).

When used in conjunction with pre-treatments which render the orthocortex more susceptible, enzymic hydrolysis with proteolytic enzymes preferentially attacks the orthocortex. This has been used as a method of separating ortho- and paracortex. The following pre-treatments have been employed: reduction and coupling of disulphide bonds with thioglycollic acid and ethyl bromide; and preferential supercontraction of the orthocortex by urea/bisulphite solution, or by heating with water (120-130°C), formamide, or phenol.

Enzymes used both with and without chemical pre-treatments have been used to produce isolated ortho- and paracortical cells from the fibre, often with the assistance of some mechanical means of breakdown; frequently there is obtained a residue of some paracortex from which not all the paracortical cells have been liberated (Dusenbury and Jeffries, 1955; Dusenbury and Menkart, 1955; Fraser and Rogers, 1953, 1955a; Golden, Whitwell, and Mercer, 1955; Kulkarni, Robson, and Robson, 1971; Laxer and

Whewell, 1955; Laxer, Whewell, and Woods, 1954; Lockart, 1960; Mercer, 1953, 1954 a & b; Mercer, Golden, and Jeffries, 1954).

1(e). Procedures for separating orthocortex from paracortex.

Golden, Whitwell, and Mercer (1955) obtained a tryptic digest of orthocortex separated from residual wool, after tryptic digestion of wool supercontracted with water at 130°C.

With a chloral hydrate density gradient, Horio, Kondo, Sekimoto and Funatsu (1965) separated the lighter orthocortical cells and the heavier paracortical cells which had been obtained by grinding fibres previously heated in water at 170°C.

Bartulovich (1964) and Ward and Bartulovich (1964) separated on a chloral hydrate density gradient ortho- and paracortical cells previously obtained by mechanically disintegrating wool cooled to the temperature of liquid nitrogen.

Partial hydrolysis, often with heating, of the fibre with 6N hydrochloric acid enabled Daveloose, Mazinque, and van Overbeke (1960), Derminot (1958), Derminot and Leveau (1956), Leveau 1956 a & b, 1957, 1958, 1959 a, b, c, d, e), Lundgren (1955), Miro (1961), Miro and Blade (1965), Parisot, Allard, and Baures (1965), Schoberl (1960), Simmonds and Bartulovitch (1958), and Ward and Bartulovitch (1955, 1956) to obtain mixtures of ortho- and paracortical cells which they later separated on a chloral hydrate density gradient.

Bradbury, Chapman, and King (1967), Chapman (1967), Chapman and Bradbury (1968), and Bradbury, Ley, and Peters (1971) used preferential deposition of gold in the paracortex, followed by ultrasonication of the fibre in formic acid, before separating ortho- from paracortical cells on an ethanol/carbon tetrachloride density gradient.

Kulkarni, Robson, and Robson (1971) separated lighter orthocortical and heavier paracortical cells by layering the cells on a mixture of carbon tetrachloride and xylene; they first liberated the

cells from the fibre by tryptic digestion followed by ultrasonication.

A number of these earlier workers reported amino-acid analyses of ortho- and paracortex, but as the separation procedures were often very degradative, not much reliance can be placed on their analyses. Chapman (1967) and Chapman and Bradbury (1968) have compared these analyses and have found little consistency among the results. Yet these crude analyses have one thing in common: they show that the paracortex is richer in cystine than the orthocortex.

1(f). Interpretation of treatments which show up the bilateral differentiation of the fibre.

Since the experimental section of this chapter is devoted to bilateral staining of wool with gold, we shall give most attention to bilateral staining treatments, especially those using metal stains.

If we hope to explain the above results we must recognise two factors each capable of influencing the outcome of any given treatment on the fibre.

The first factor is one of differences between the ortho- and paracortex in structural organisation: the orthocortex contains much more inter-macrofibrillar material; the nuclear remnants are larger in the paracortex; and, though the exact ratios of microfibrils to matrix in the two segments are disputed, it is very probable that the paracortex contains significantly more highly cross-linked matrix.

The second factor is one of differences in chemistry between microfibrils and matrix: in view of the different proportions of microfibrils and matrix in the two segments, such differences in chemistry can be very significant. Thus if the matrix is especially rich in certain amino-acids, those amino-acids must be important in the paracortex, the segment containing more matrix. Similarly, if microfibrils are especially rich in certain other amino-acids, those amino-acids must be more important in the orthocortex, the segment containing more microfibrils.

The higher proportion of matrix (which stains darker than microfibrils with osmium) in the paracortex, is obviously responsible for

the darker background seen in that segment in Figure 1 of the paper by Rogers and Filshie (1963).

Thus preferential staining of matrix rather than microfibrils, when accompanied by preferential staining of para- rather than orthocortex, is good evidence for a higher proportion of matrix in the paracortex. Similarly, preferential staining of microfibrils rather than matrix, when accompanied by preferential staining of ortho- rather than paracortex, is also good evidence for a higher proportion of microfibrils in the orthocortex. Both these situations are represented among the examples of metal staining we have discussed.

The staining with uranyl acetate is a neat example. This reagent stained microfibrils and orthocortex preferentially (other evidence indicated its specificity for carboxyl groups); after conversion of the cystine present to S-carboxymethyl groups, the reagent stained the matrix and the paracortex preferentially.

Consistent with this picture is the preferential staining of the matrix accompanied by preferential staining of the paracortex, in both normal and S-carboxymethylated wool treated with ammoniacal silver nitrate. This result, together with the lack of increased uptake of silver on S-carboxymethylation of the wool, rules out carboxyl groups as possible binding sites for silver: sulphur is implicated instead (Kassenbeck, 1967). A similar mechanism for silver binding may therefore account for the preferential staining of the paracortex with silver nitrate and silver acetate. On the other hand Simpson and Mason (1969) found that mainly carboxyl groups, and only a little cystine, bind to silver ions in silver stained wool.

The preference of mercury for the paracortex can be explained by the well known affinity of mercury for sulphur, which is of course present in greater amount in the paracortex (where the content of matrix is greater).

The presence of precipitates also implicates sulphur, as mercuric sulphide is a recognised precipitate.

The precipitation of thallium sulphide occurring on staining of the paracortex with thallium carbonate likewise correlates with the greater content of sulphur in the paracortex.

Accessibility is no doubt the factor responsible for the staining of the orthocortex in fibre cross-sections post-stained with the very large ions of phosphotungstic and phosphomolybdic acid. The smaller amount of cross-linked matrix and the much larger amount of non-keratinous inter-macrofibrillar material in the orthocortex explain this effect.

The affinity of gold for the paracortex (apart from elemental gold deposited by formic acid), as observed by Chapman (1967) and Chapman and Bradbury (1968), is probably due to the higher content of cystine in the paracortex (in the matrix); (see also Figure III-4 of this thesis).

Dark crystallites of lead sulphide appear to be accommodated in the large nuclear remnants of the paracortex of fibres.

Chapman and Bradbury (1968) and Kulkarni, Robson, and Robson (1971) determined that the orthocortex contained 26% and 48% (respectively) more tyrosine than the paracortex. This agrees well with the theory that staining of the orthocortex with metal ions from acidic nitrite solution is due to complexing of the ions with tyrosine.

Binding of metal ions to polar groups has been invoked in cases of preferential staining of microfibrils rather than matrix, and we have noted that in these cases preferential staining of the ortho- rather than the paracortex occurs. Now the work of Kulkarni et al. shows that for both ortho- and paracortex there is as much as several times the amount of polar residues in the low-sulphur protein fraction as in the high-sulphur protein fraction. Furthermore, binding of metal ions to disulphides, or to the carboxyl ends of S-carboxymethyl groups produced from disulphides, has been invoked in cases of preferential staining of the matrix, and this

staining has been accompanied by preferential staining of the paracortex. The work of Kulkarni et al. shows that for both ortho- and paracortex there is almost three times the amount of cystine in the high-sulphur fraction as in the low-sulphur fraction. So if we accept that the low- and high-sulphur fractions comprise the microfibrils and matrix respectively, then, since the paracortex contains more matrix than the orthocortex and since the orthocortex contains more microfibrils than the paracortex, we have a very attractive explanation of staining with metal stains and organic dyes.

Acid and basic dyes stain the orthocortex preferentially; the orthocortex is richer in microfibrils; the microfibrils (according to the theory) are very rich in polar groups; therefore both acid and basic dyes form an equilibrium association with the polar groups of the orthocortex. Furthermore dye molecules commonly have large dimensions, and so accessibility to the matrix (of which there is more in the non-dyeing paracortex) may be restricted. Accessibility is also conferred on the orthocortex by its inter-macrofibrillar material. Oxidation or S-carboxymethylation of the fibre allows dyeing of the paracortex by methylene blue on two counts: hindrance to the dye molecules by the cross-links is removed to a greater extent in the paracortex since that segment originally contained more matrix; there is a relatively greater increase in sulphonic acid or S-carboxymethyl groups (to which the basic dye molecules can bind) in the paracortex owing to the greater original content of matrix in that segment. Finally the bilateral dyeing of cross-sections of fibres with organic dyes proves that the differentiation produced is not due to the relative permeability of the cuticle on the ortho- and paracortical sides of the cortex.

Under hydrolytic conditions which do not break disulphide bonds, the more highly cross-linked structure in the paracortex probably restricts hydrolytic breakdown (Kulkarni et al. 1971). Thus the relative stability

of the paracortex may be due to the difficulty reagents experience in entering the high proportion of highly cross-linked matrix in this segment, and to the difficulty of removing through the matrix peptide fragments which could also remain linked to the structure via disulphide bonds. In the orthocortex the inter-macrofibrillar material no doubt facilitates the access of reagents, especially since this material is itself removed by hydrolysis (Leach et al. 1964).

Preferential supercontraction of the orthocortex could indicate contraction of alpha-helices, which are present in greater amount in the orthocortex.

Swelling and dissolution of the paracortex on oxidation of the fibre with bromine can be accounted for by the relatively larger production of sulphonic acid groups in that segment (due to the larger original cystine content); the resultant repulsion between charged sulphonic acid groups is therefore greater in the paracortex, and so this segment preferentially swells.

The liberation of ortho- and paracortical cells on acid hydrolysis of the fibre, achieved by workers already mentioned, is consistent with the breakdown of the cell membrane complex in both the ortho- and paracortex during hydrolysis, as observed by Leach et al. (1964).

Enzymic dissolution of the orthocortex of wool previously subjected to treatments which break disulphide bonds, is explained by the higher cystine content initially in the paracortex. If not all the disulphides in the fibre are broken, the paracortex must still contain cross-links even at the stage where the cross-links of the orthocortex have all been removed. The remaining cross-links in the paracortex prevent its attack by enzymes. This explanation agrees with the finding that the paracortex is also attacked by the enzyme if the pre-treatment is not sufficiently mild (e.g. Dusenbury and Menkart, 1955).

The incomplete separation of paracortical cells from the

paracortex found by Kulkarni et al. (1971) after ultrasonication of wool

previously digested with trypsin, was considered by them to be due to the

greater ease of penetration of the trypsin into the intercellular material

of the orthocortex, compared to the ease of penetration into the

intercellular material of the paracortex. This greater access can be

explained both by the lower cross-linking in the orthocortex, and by the

greater amount of inter-macrofibrillar material in that segment. The

electron microscopy of these workers shows extensive attack of this inter-

macrofibrillar material by the trypsin, and so the trypsin can gain access

to the orthocortical intercellular material once the inter-macrofibrillar

material is removed.

and this percentage is needed in calculating the quantities used in the

recipe, since the formula weight of chloroauric acid is uncertain owing

to the unknown proportion of water of crystallization in the crystal lattice.

In this thesis the solution will be referred to as "2% gold chloride". As

made up, this solution has a pH of 1.37.

2(c). Staining of wool with gold.

In a number of cases wool was stained with gold by the method,

due to Laxer and Rose (1954), now described. 1 gm of wool was immersed in

100 ml of 99% formic acid for 20 minutes, rinsed in demineralised water for

2 minutes, and then blotted dry on filter paper. The wool was then placed

in 100 ml of 2% gold chloride solution for 10 minutes, rinsed in demineralised

water to remove gold chloride solution on the fibre surface, and blotted dry

on filter paper. After this the wool was immersed in 100 ml of 25% formic

acid for 4 hours and was finally rinsed thoroughly in demineralised water to

remove as much as possible of the formic acid.

It was found that during the immersion in gold chloride, air

pockets trapped in felted regions of the fibre mass prevented the solution

from reaching parts of the fibre, and resulted in uneven staining, unless

2. Materials and Methods

2(a). Cleaning of wool.

The wool used was Merino 64's. It was cleaned by the procedure described in Section 2(a) of Chapter II.

2(b). Preparation of gold chloride solution.

2% gold chloride solution was prepared by dissolving 6 gm chloroauric acid ($\text{HAuCl}_4 \cdot x\text{H}_2\text{O}$) in 220.2 ml of water. Gold chloride, strictly auric chloride, AuCl_3 , does not furnish Au^{3+} ions when it is dissolved in water; instead the gold is bound in a complex anion, probably AuCl_3OH^- (Kolthoff and Elving, 1966). The above recipe gives a solution having the same concentration of gold as there would be in a hypothetical 2% AuCl_3 solution. The chloroauric acid used had a nominal gold content of 49%, and this percentage is needed in calculating the quantities used in the recipe, since the formula weight of chloroauric acid is uncertain owing to the unknown proportion of water of crystallisation in the crystal lattice. In this thesis the solution will be referred to as "2% gold chloride". As made up, this solution has a pH of 1.37.

2(c). Staining of wool with gold.

In a number of cases wool was stained with gold by the method, due to Laxer and Ross (1954), now described. 1 gm of wool was immersed in 100 ml of 99% formic acid for 20 minutes, rinsed in demineralised water for 2 minutes, and then blotted dry on filter paper. The wool was then placed in 100 ml of 2% gold chloride solution for 10 minutes, rinsed in demineralised water to remove gold chloride solution on the fibre surface, and blotted dry on filter paper. After this the wool was immersed in 100 ml of 25% formic acid for 4 hours and was finally rinsed thoroughly in demineralised water to remove as much as possible of the formic acid.

It was found that during the immersion in gold chloride, air pockets trapped in felted regions of the fibre mass prevented the solution from reaching parts of the fibre, and resulted in uneven staining, unless

the following precautions were adopted. Felting was reduced by first chopping the wool with scissors to lengths of about 0.25 cm.; about 1.5 gm of intact wool was chopped and 1 gm of the chopped fibre was used for staining. Uneven staining due to air pockets was further reduced by connecting the flask containing the fibres and the gold chloride solution to a water pump, and then alternately exhausting the air in the flask and releasing the vacuum, during the 10 minutes immersion time.

All ash determinations were performed by a microanalytical service.

Figure III-3 shows the ash content, expressed as a percentage of the total fibre weight, in samples of gold stained wool after increasing periods of immersion of sample in gold chloride. The three curves represent three different methods of staining the wool with gold, as now described. Wool was immersed in a dye bath of 2% gold chloride (liquor to wool ratio of 100 : 1) at pH 1.37. After the required time had elapsed some of the wool was withdrawn and rinsed for 2 minutes in water to remove all the solution from the surface of the fibres. This wool was then divided into three portions, the first of which was dried and then analysed for its ash content. The second portion was immersed in 25% formic acid for 4 hours, washed in water, dried, and analysed for ash. The third portion was immersed in several changes of water for 2 weeks, dried, and analysed for ash. The procedure was repeated for other wool samples withdrawn from the dye bath after different periods of time, and the percentages of ash contents plotted on the three curves. Only a portion of each sample prepared for analysis was analysed, the remainder being set aside.

2(d). Sectioning of fibres for light microscopy.

A Hardy microtome was used to prepare cross-sections of gold stained fibre for examination in the light microscope. Gold stained fibres which were to be sectioned were not chopped finely prior to staining because it was almost impossible to align such short lengths of fibres correctly in

The fibres were placed in 95% formic acid and subjected to the action of the slot of the microtome. The fibres were embedded by racking up the bundle of aligned fibres in the slot of the microtome, and then applying a smear of medicinal "plastic skin" to their protruding ends. The embedding material set in a fraction of a minute. Each section was cut with an unused razor blade clamped in a razor blade holder which allowed excellent control of the blade.

2(e). Electron microscopy.

Short lengths of gold stained fibres from each sample to be investigated were aligned in a bundle, both ends of which were tied with cotton. This bundle was embedded in Araldite in a gelatin capsule in a similar manner to that described for the embedding of cuticle in Section 2(f) of Chapter II. The only staining given to the fibres before sectioning was the gold staining treatment, which varied from sample to sample as indicated in Table III-2. The thin sections shown in Figures III-4, III-5, and III-6 were post-stained with lead acetate. The thin section shown in Figure III-7 was not post-stained with this reagent, as sufficient heavy metal had been deposited in the fibre by the exceptionally prolonged staining in gold chloride. This thin section in Figure III-7 was taken from a sample of fibres stained by Mr. D.E. Peters.

The gold stained cortical cells referred to in Section 3(a) were aligned and embedded by a specially developed technique which was fundamental to the purpose of the experiment conducted, and so this technique has been described in Section 3(a).

Mr. D.E. Peters cut all the thin sections of both fibres and cortical cells, using an L.K.B. Ultratome, and performed all post-staining of sections, and finally operated the Hitachi Model HU-IIC-S electron microscope.

2(f). Preparation of cortical cells.

Gold stained and unstained cortical cells were prepared from gold stained and unstained fibre respectively by the following method.

The fibres were placed in 99% formic acid and subjected to the action of a Polytron cutter for 20 minutes. This released from the fibre vast quantities of cortical cells, owing to the mechanical action and also to the dissolution of intercellular cement by the formic acid. Light microscopy showed that the formic acid contained numerous cortical cells chopped into fragments, but also vast quantities of intact cortical cells, numerous clusters of two or three cortical cells joined end to end, severely damaged fibres containing cortical cells which had not been completely detached from the fibre, and fragments of cuticle cells. The suspension of formic acid was ultrasonicated for 5 minutes to detach completely the loosened cortical cells still in the fibre, and to separate the cortical cells present in the clusters from each other. The large fibres were removed by filtration of the suspension through a coarse wire sieve. The material was then transferred to ethanol at the centrifuge using four washings of ethanol.

Cortical particles were obtained by passing the ethanolic suspension through a Nytrex nylon filter cloth of pore size equal to 120 microns, and then passing the resultant filtrate through a Nytrex filter cloth having a pore size of 50 microns. The material retained on the second filter was collected. Skin flakes, cuticle fragments, and most fragments of chopped cortical cells passed through the 50 micron filter. Considerable numbers of intact cortical cells also passed through this filter, probably by entry of the cells end on into the filter pores. (Attempts to salvage these intact cells with a 25 micron filter furnished a mixture of intact cortical cells contaminated with large cortical fragments). To ensure the purity of the preparation, both filtrations through the 120 and 50 micron filters were actually performed twice, and the ethanolic suspensions were ultrasonicated for 10 seconds immediately before filtration so as to disperse aggregated particles. The resultant sample of cortical cells was stored in ethanol.

2(g). Fractionation using density gradients.

Linear carbon tetrachloride/ethanol density gradients were prepared using the apparatus described by Chapman (1967). The essential arrangement of this apparatus was such that 15 ml of the less dense liquid was gradually mixed with the denser liquid in a chamber which originally contained 15 ml of the denser liquid, and which had at its bottom a fine orifice to allow the passage from the apparatus of liquid of increasing density. The 30 ml of liquid passed were collected in a 40 ml centrifuge tube. An ethanolic suspension of the mixture of particles to be fractionated was layered as a narrow band onto the top of the gradient, after first ultrasonicated the suspension for 10 seconds to disperse aggregated particles.

An ethanolic suspension of a mixture of gold stained cortical cells (prepared with the Polytron from wool stained by the method of Laxer and Ross) was ultrasonicated for 10 seconds and then layered on a density gradient whose limits of density were 1.287 and 1.320 gm/cc. Centrifugation overnight at 2500 rpm produced a narrow light pink band of cortical cells in the middle of the gradient, and a sediment at the bottom of the tube, of dark pink cortical cells. This sediment was resuspended in ethanol and was washed free of carbon tetrachloride. The ethanolic suspension of this sediment was ultrasonicated for 10 seconds, then layered as a band on a carbon tetrachloride/ethanol gradient whose limits of density were 1.310 and 1.356 gm/cc. After centrifugation overnight at 2500 rpm, a thin layer of light pink cells remained on top of the gradient and a sediment of dark pink cells was found at the bottom of the tube. Evidently the thin pink layer belonged to the fraction obtained as a band in the first gradient, and had been aggregated with the sediment in that gradient, for when these cells were resuspended in ethanol and layered on a gradient of the first kind, and then were centrifuged, they appeared in the middle of the gradient in the same position as the narrow band of pink cells occurred before.

Ultrasonication of the sediment of the first gradient prior to layering on the second gradient had detached these contaminating cells. The dark sediment of the second gradient could not be separated into further bands by using carbon tetrachloride/ethanol gradients of greater density.

This sediment was obtained as a band with a wide density range in the region of 1.5 to 1.7 gm/cc when it was layered on an ethylene dibromide/ethanol gradient. No sediment was produced at the bottom of this gradient, and so the sediment obtained at the bottom of the gradient of density 1.310 to 1.356 gm/cc represented a pure fraction.

As cortical cell fractions of unequivocal purity were required for the experiment in Section 3(a), the fractions used were obtained as follows from carbon tetrachloride/ethanol density gradients. The light fraction was taken from the band appearing after centrifugation in the middle of a gradient of density 1.287 to 1.320 gm/cc, after a mixture of the light and heavy cells had been layered on to the gradient. The heavy fraction was taken from the sediment appearing after centrifugation at the bottom of a gradient of density 1.310 to 1.356 gm/cc, after a mixture of the light and heavy cells had been layered on to the gradient: the layer of light cells remaining on top of this gradient after centrifugation was not used. The four solutions used to make the two gradients had the following compositions:

gm/cc	1.287	1.320	1.310	1.356
ml CCl_4	30.97	32.99	32.38	35.20
ml EtOH	19.04	17.01	17.63	14.80

To decide unequivocally whether such a separation is possible, cross-sections of cortical cells of both kinds were examined in the electron microscope and compared with ortho- and paracortical cells seen in gold stained fibres in the electron microscope. The alignment of spindly

3. Results and Discussion

3(a). Vindication of separation procedure for obtaining orthocortical and paracortical cells.

Ortho- and paracortical cells from Merino 64's fibres had earlier been separated in this laboratory, and the amino acid analysis of both cortical cell fractions and of the high-sulphur and low-sulphur proteins of both fractions had been performed (Bradbury, Chapman, and King, 1967; Chapman, 1967; Chapman and Bradbury, 1968). The separation procedure involves staining the paracortex preferentially with gold (Laxer and Ross, 1954) and disrupting the fibre mechanically in formic acid. After using a screening procedure to obtain a clean sample of cortical cells free from cuticle and from incompletely separated fibrous components (Bradbury and Chapman, 1964), the more heavily stained dark red paracortical cells can be separated from the less heavily stained light pink orthocortical cells by centrifugation in a density gradient (Chapman and Bradbury, 1968). Since gold in the bilaterally stained fibres can be seen by both light and electron microscopy to be deposited preferentially in the paracortex, and since after disruption any residual fibres still retain their bilateral staining, it has been assumed that of the cortical cells liberated, the more heavily stained cells do in fact originate in the paracortex, and the less heavily stained cells in the orthocortex. Now colloidal gold is produced in the formic acid during disruption of the fibre. It was just possible then that the difference in gold content of the two types of liberated cortical cells is due simply to cells losing different amounts of gold during the disruption, and that separation into ortho- and paracortical cells cannot therefore be achieved by this method.

To decide unequivocally whether such a separation is possible, cross-sections of cortical cells of both kinds were examined in the electron microscope and compared with ortho- and paracortical cells seen in gold stained fibre in the electron microscope. The aligning of spindly

cortical cells by stroking them in viscous Araldite on a Perspex microscope slide which, after the Araldite had hardened, could be cut up and embedded in more Araldite, allowed sections to be then cut perpendicularly through vast numbers of well aligned cells. (The excellent alignment and frequency of occurrence of the cells was checked under the light microscope before the slide was cut up). Thus one could scan and compare numerous cortical cell cross-sections in the electron microscope.

Figures III-1 and III-2 show cross-sections of cortical cells from the light and heavy fractions respectively. The sections have been post-stained with lead citrate and each is representative of the fraction from which it came. The cortical cell in Figure III-1 originates from the orthocortex as shown by the typical appearance of the small individual macrofibrils (Rogers, 1959b; Bones and Sikorski, 1967). The cortical cell in Figure III-2 does not exhibit subdivision into small macrofibrils in the manner of an orthocortical cell, but has a large nuclear remnant filled with gold particles (Chapman and Bradbury, 1968) and a more visible microfibril/matrix structure than that of the cortical cell in Figure III-1. The cell in Figure III-2 is therefore a paracortical cell (Rogers, 1959b). Approximately 800 sections from the light and heavy fractions were examined and in greater than 99% of the cases the light fraction consisted of orthocortical cells and the heavy fraction of paracortical cells. Thus the separation procedure for obtaining ortho- and paracortical cells from fine wool is fully vindicated. A note on this work has recently been published (Bradbury, Ley, and Peters, 1971) and a copy is bound in at the back of this thesis.

3(b). The cause of bilateral staining in gold stained wool fibre.

Figures III-4 to III-7 depict electron micrographs of cross-sections of gold stained wool fibres. The conditions of gold staining varied in each sample, as indicated in Table III-2 which also compares the

in fibres of two samples of this series unmistakable bilateral

essential features of the micrographs. All the sections except that in Figure III-7 were post-stained with lead acetate.

Figure III-3 shows the increase in ash content of wool samples stained for increasing periods in 2% gold chloride at pH 1.37 and at room temperature. (The ash content in gold stained wool is effectively the same as the gold content, since the ash content of virgin wool is negligible). The three curves represent three different methods of staining: (1) immersion in 2% gold chloride only; (2) immersion in 2% gold chloride followed by immersion in 25% formic acid for 4 hours ("formic acid series"); (3) immersion in 2% gold chloride followed by immersion in several changes of water for 2 weeks ("water washed series"). In each method the fibres were rinsed for 2 minutes in water, immediately after immersion in gold chloride, to remove any gold chloride solution adhering to the surface of the fibres. All the samples treated only with gold chloride had a dirty yellow or khaki colour after immersion in the reagent. All the samples washed for an extended period in water after the gold chloride treatment slowly changed in colour from khaki to a rose-purple during the water wash. The colour of the samples immersed in 25% formic acid after the gold chloride treatment, changed to purple during the immersion, but of these the samples which had had the longer treatment in gold chloride were less deeply coloured and had more of a pale rose-purple hue.

Table III-3 indicates the appearance of samples from the "formic acid series" and the "water washed series". In the light microscope the samples were examined for bilateral gold staining in the fibres.

In the "formic acid series" fibres of samples treated with 2% gold chloride for up to 40 minutes were dark purple to the eye, and in the microscope they appeared dark red-brown and showed bilateral differentiation of this colouring along their length. Gold chloride treatments of 5 and 40 minutes respectively, followed by immersion in formic acid, produced in fibres of two samples of this series unmistakable bilateral

differentiation of the stain in the fibre cross-sections. However, in this series fibres from the remaining samples which had had up to 36 hours in 2% gold chloride, followed by immersion in formic acid, were only a pale rose-purple to the eye, and in the microscope these fibres appeared almost colourless and so no bilateral staining was visible.

The fibres of the samples of the "water washed series", though rose-purple to the eye, appeared pale red-brown or almost colourless in the microscope and showed no bilateral staining along their lengths. In this series, cross-sections of fibres which had been treated with 2% gold chloride for 20 minutes and 24 hours, and which had then been washed in water for 2 weeks, showed no bilateral staining.

Table III-4 shows the ash content of some gold stained wool samples before and after the attempted desorption of gold using sulphuric acid.

Table III-5 shows the effect of the mechanical disruption of the fibre on the bilateral differentiation.

Figures III-4 to III-7 show that in gold stained fibre, gold can be present in three forms, as found by Chapman and Bradbury (1968). In Figure III-4, which shows a cross-section of a fibre stained by the method of Laxer and Ross (10 minutes being the time of immersion in 2% gold chloride), a histochemical staining causes the background in the paracortex to be darker than the background in the orthocortex. Gold is also present in the form of large particles in the nuclear remnants, especially in the large nuclear remnants of the paracortex. Gold is present in yet another form, namely as smaller particles of gold distributed throughout the whole cortex, but concentrated very obviously in the paracortex.

The sizes of the two kinds of particles are similar to the sizes observed by Chapman and Bradbury, which were 20-80 angstroms for the smaller kind and 100-400 angstroms for the larger kind. The particles

cannot be derived from lead acetate since they were still observed in sections not post-stained with this reagent. Chapman and Bradbury postulated that the particles resulted from reduction of Au(III) by the formic acid used. The size of the particles lies within the size distribution of colloidal gold particles in red to purple colloidal gold solutions (Mellor, 1923), and the purple colour of fibres stained by the method of Laxer and Ross only develops during the immersion of the gold treated fibres in the 25% formic acid. Furthermore no such gold particles are visible in the cross-section of the fibre treated with gold chloride only (Figure III-7). Since the mixing of 2% gold chloride at pH 1.37 with 25% formic acid does not produce a colloidal gold solution, it seems that particles can only be produced in the fibre if material from the fibre itself participates in the reduction reaction.

Since there is more matrix in the paracortex, the preferential histochemical staining of this segment is consistent with the reduction of the Au(III) of the gold chloride solution by the cystine disulphides to form isolated atoms of elemental gold. The preferential staining is also consistent with the entry of additional Au(III) into the matrix, after swelling of the matrix caused by oxidation of the disulphides to charged sulphonic acid groups which can repel each other. We recall that Menkart and Coe (1958) produced swelling of the paracortex on oxidation with bromine; and that Rogers (1959b) found, in reduced wool treated with osmium tetroxide, a large additional uptake of osmium which could not be accounted for by the reaction of osmium tetroxide with sulphhydryls, and which could only be explained by the accessibility of the matrix under the conditions used.

Omission of the initial treatment of the fibre in 99% formic acid prior to staining has only a small effect (Figure III-5). There are fewer and somewhat smaller particles in the nuclear remnants than in the remnants in Figure III-4. Preferential background histochemical staining

in the paracortex, and preferential deposition in the paracortex of the smaller kind of gold particle, occur as in Figure III-4.

Washing of the fibre for an extended period in water, immediately after immersion in 2% gold chloride solution but prior to treatment with formic acid (Figure III-6), prevents the formation of large particles of gold in the nuclear remnants, presumably by removing from these regions any Au(III) which would otherwise be available for reduction by the 25% formic acid.

Also, no histochemical staining of the nuclear remnants occurs under these conditions. Yet in the rest of the cortex (a region surely as accessible to water as the nuclear remnants are during the extended wash) the background histochemical staining remains and the paracortex is preferentially stained histochemically as in Figures III-4 and III-5.

Unlike the situation in Figures III-4 and III-5, the deposition of the smaller gold particles in Figure III-6 is not so obviously concentrated in the paracortex but is instead distributed more evenly across both segments. This suggests a slow transfer of Au(III) of the gold chloride solution across the ortho/para junction during the long wash in water, and then reduction of this Au(III) to give the small gold particles. The mechanism producing the large particles in the nuclear remnants in Figures III-4 and III-5 must be unlike the mechanism producing the small particles in Figures III-4, III-5, and III-6, because washing with water removes the larger but not the smaller particles.

The complete absence of gold particles, large and small, in the sample for which the reduction step in 25% formic acid was omitted (Figure III-7: 2½ months in 2% gold chloride, pH 4.5) implies that all such particles are produced by the reducing action of formic acid. The treatment used on this sample is distinguished also by the very much longer period in gold chloride (2½ months as compared to 10 minutes in Figures III-4, III-5, and III-6), and by the pH used of 4.5 instead of

1.37 as in the other samples. Unlike the other samples, nuclear remnant material in this case has a dark histochemical staining, and the orthocortex has a much darker histochemical staining; both these features must be attributed to the extensive time of staining. But the really remarkable feature of Figure III-7 is the degradation which has occurred in the paracortex; the voids created in this segment contrast with the dark staining of the orthocortex. It seems that during the 2½ months of immersion extensive oxidation of disulphide bonds has occurred, and as the paracortex contains much more matrix, removal of material from this segment has taken place. Hydrolysis of peptide bonds, during the long immersion at acidic pH, could have facilitated this removal. In contrast, only a small fraction of the disulphides would have been oxidised in Figures III-4, III-5, and III-6, since the analyses of Chapman and Bradbury (1968) showed that in wool stained by the method of Laxer and Ross (for which the time of immersion in 2% gold chloride was 10 minutes), only about 16% of the original cystine was oxidised to cysteic acid.

We note from Figure III-3 that the percentage of gold in fibres stained by the three different procedures reaches an equilibrium value in each case: about 26% for immersion in gold chloride only; about 33% for the "formic acid series"; and about 14% for the "water washed series". However the three methods of staining result in significant differences in gold content only when the times of immersion in 2% gold chloride (pH 1.37) are greater than about 1½ hours.

The samples immersed in 2% gold chloride solution only were just given a brief wash in water for two minutes (to remove gold chloride solution from the fibre surface) before drying prior to being analysed for ash. Part of their gold content was therefore due to the presence of gold chloride solution in the fibre. The levelling off of the ash content with longer times of immersion in gold chloride thus represents both the maximum accommodation of gold chloride solution within the fibre and the

maximum reaction of Au(III) with the fibre. Fibres from samples from the plateau region of the curve had a degraded appearance and harshness of handle, and we recall the appearance of Figure III-7.

The lower gold content of fibres washed extensively in water immediately after prolonged immersion in gold chloride, compared to the gold content of unwashed fibres, can be explained by diffusion of the gold chloride solution out of the fibre; the several changes of water used facilitated this diffusion. The residual gold is gold which has reacted with the fibre.

The curves representing the "water washed series" and immersion in gold chloride only, coalesce at short times in gold chloride; also washing in water after 10 minutes immersion in gold chloride prevents the deposition of gold particles in the nuclear remnants on treatment with 25% formic acid (Figure III-6). Therefore these gold particles cannot comprise much of the total gold content of the fibres in which they are present.

Samples immersed in gold chloride and then washed in water, slowly developed a rose-purple colour during the wash. This was surprising since colour had only been previously observed on development with formic acid, and fibres treated only with gold chloride did not develop colour. Perhaps some of the Au(III) inside the fibre, in the gold chloride solution slowly being replaced by water, was slowly reacting with the fraction of cystine which had not been oxidised during the immersion in gold chloride. The slow rate of reduction to elemental gold particles of colloidal size could be explained by the progressive dilution, also occurring within the fibre, of the gold chloride solution.

To explain the surprising result that treatment in 25% formic acid after gold staining actually increases the proportion of gold over that present if no formic acid is used, it is necessary to assume that the formic acid removes protein from the fibre. This is quite plausible since all fibres subjected to a prolonged treatment in gold chloride had a considerably degraded appearance and a harshness of handle not found in

untreated wool, and no doubt extensive oxidation of disulphides and some hydrolysis of peptide bonds had occurred. Peptide fragments would then have been released in large amounts by the formic acid, as formic acid is an excellent swelling reagent for wool. For short times of immersion in gold chloride, no degradation of the fibres was visible to the eye, and therefore when the fibres were placed in formic acid, a negligible quantity of material was released. Hence the curves for the "formic acid series" and for immersion in gold chloride only, coalesce at short times in gold chloride.

It is likely that in the "formic acid series", immersion in 25% formic acid for 4 hours had a similar effect to immersion in water for 2 weeks, and that gold chloride solution inside the fibre was removed. This would have decreased the gold in the fibre, but apparently removal of protein more than compensated for this and the resultant percentage of gold increased.

From Table III-3 we see that in the "formic acid series", for times of up to 40 minutes in gold chloride, fibre cross-sections showed the bilateral staining typical of the Laxer and Ross treatment (in which immersion of only 10 minutes in gold chloride was used). But if after up to 40 minutes in gold chloride, the fibres were washed for an extended period in water instead of being immersed in formic acid, then the fibre sections were only a very pale red-brown in the light microscope and showed no differentiation in staining. Now an electron micrograph of a similarly treated fibre (Figure III-6) shows that the large particles in the nuclear remnants have been removed. Thus we have evidence that the bilateral staining seen in the light microscope is due to gold particles in the nuclear remnants. Figure III-4 shows that particles in the remnants of the paracortex are larger and occur more often than those in the remnants of the orthocortex. The colour of fibres washed in water (rose-purple to the eye and pale red-brown in the light microscope) is no doubt due to the

smaller gold particles, seen distributed evenly over both segments in Figure III-6, whose size (50-100 angstroms) is at the lower end of the size distribution of colloidal gold particles in coloured colloidal gold solutions (Mellor, 1923). Note that a bilaterally deposited background histochemical stain (Figure III-6), if due only to isolated gold atoms produced by oxidation of disulphides, cannot contribute to a bilateral effect seen by light microscopy since the individual gold atoms are smaller than colloidal size.

It is curious that formic acid treatment, which produces a marked bilateral staining in fibres immersed in gold chloride for up to 40 minutes, should produce no apparent colour in the microscope and therefore no visible bilateral staining in fibres immersed in gold chloride for much longer times (Table III-3). However in the "formic acid series", although the percentage by weight of the fibre, of gold in the fibre, was higher at longer times in gold chloride owing to removal of protein from the fibre, the actual amount of gold present may have been less than at shorter times in gold chloride, owing to the gradual replacement of gold chloride solution inside the fibre by formic acid. In the fibre, there would therefore have been much less gold chloride solution available for reduction, and so fewer colloidal particles would have been produced. This would explain why the colour of the fibre was not as deep. In the microscope, lack of visible colour in samples pale rose-purple to the eye, is due simply to the intensity of light transmitted in the microscope up through the sample.

The data from electron microscopy (Figures III-4, III-5, and III-6) and from light microscopy (Table III-3) indicate that the bilateral gold staining seen in the light microscope is due to the presence of more gold in the paracortical nuclear remnants than in the orthocortical remnants, and also to the preferential deposition of the smaller particles in the paracortex. However the coalescence of the curves, at short times in gold chloride, for the "water washed series" and for the immersion in gold

chloride only, when considered with Figure III-6, shows that the gold particles in the nuclear remnants amounted to a negligible proportion, or at least to only a small proportion, of the total gold in the fibre. For this to be true the total mass of the highly dispersed isolated gold atoms of the histochemical staining, plus the total mass of any additional gold present due to swelling of the matrix (and which would probably also contribute to the histochemical staining), plus the total mass of the dispersed small gold particles, must have greatly exceeded the total mass of the relatively larger but exceedingly fewer particles in the nuclear remnants.

The effectiveness of sulphuric acid in desorbing gold from a few gold stained samples is shown in Table III-4. The ash contents of samples A, B, and D (10.8%, 10.2%, and 10.3% respectively) were within experimental error. Sulphuric acid has caused a 25% decrease in the gold content of both samples A and B. In sample A there cannot have been present any gold particles of colloidal size since Figure III-7 shows no such particles in a fibre treated with gold chloride solution for 2½ months. The removal of gold from both samples probably cannot represent removal of gold in gold chloride solution inside the fibre, since washing Sample A in water for 2 weeks did not remove any gold. This suggests that all the Au(III) in the gold chloride solution which entered the fibre in the first 40 minutes (Sample A), had reacted with fibre. Now we have postulated reaction of Au(III) with disulphides to give isolated atoms of elemental gold. It is unlikely that the decrease in gold content caused by the sulphuric acid was due to removal of these gold atoms, since gold can only be mobile if present as Au(III) in a complex anion, and sulphuric acid cannot oxidise elemental gold to Au(III). Probably the gold remaining after desorption by sulphuric acid is this elemental gold. There is therefore a need to postulate a different kind of reaction as well as the reaction of Au(III) with disulphides. Some gold must be bound in a form which can be decomplexed with sulphuric acid.

Simpson and Mason (1969) have found evidence for the binding of silver to carboxyls, and they were able to desorb Ag(I) from silver stained wool by immersing the sample in sulphuric acid: hydrogen ions from the acid competed successfully with Ag(I) for the carboxyl anions. So by analogy this author had hoped that Au(III) was complexed to carboxyls and that such Au(III) could be decomplexed with sulphuric acid.

However from nuclear magnetic resonance studies of complexing of Au(III), in gold chloride solution, with small molecules, Peters (1971) has found good evidence that no reaction of Au(III) with carboxyls occurs, but that reaction does occur with charged amine groups and with disulphides, and that the disulphides are more reactive than the amine groups towards the Au(III). He was unable to decide from his data whether the reaction of the disulphides was due to oxidation, giving sulphonic acids and elemental gold atoms, or to co-ordination of sulphur atoms of the intact disulphides bonds to the Au(III). In the complexing of Au(III) with charged amine groups, a proton was replaced by Au(III).

It is therefore probable that sulphuric acid has desorbed gold from gold stained wool by decomplexing Au(III) from amine groups, thereby restoring to those groups protons which had been replaced by Au(III).

This reaction with amines would stain microfibrils in preference to matrix, and orthocortex in preference to paracortex. However this staining is masked by the much stronger preferential staining of matrix and paracortex, due to reaction with disulphides. This is so for two reasons: the ratio of matrix in the paracortex to matrix in the orthocortex is greater than the ratio of microfibrils in the orthocortex to microfibrils in the paracortex; and the ratio of cystine in the matrix to cystine in the microfibrils is much greater than the ratio of basic groups in the microfibrils to basic groups in the matrix (Kulkarni et al. 1971).

It had been hoped that the question of whether the bilateral gold staining is a rate or an equilibrium effect, could be resolved by examining for bilateral staining cross-sections of fibres stained with increasing amounts of gold. In other words, is the differential staining due simply to gold entering the paracortex more rapidly (a rate effect, in which case the orthocortex would eventually acquire as much gold as the paracortex if the staining is prolonged); or is the differential staining still present when the fibre has acquired the maximum amount of gold possible (an equilibrium effect)?

Unfortunately in the "formic acid series" the actual amount of gold present in the samples probably decreased with increasing time in gold chloride, as already discussed, even though the percentage of gold increased. Besides, the colour in the fibres decreased with increasing time in gold chloride. Hence no samples were available which were coloured and which contained a maximum complement of gold produced by long immersion in gold chloride, and so it was impossible to decide by light microscopy whether bilateral gold staining is a rate or an equilibrium effect.

If fibres bilaterally stained with gold (by the method of Laxer and Ross) are mechanically disrupted, they furnish gold stained ortho- and paracortical cells. The difference in histological and chemical structure between ortho- and paracortical cells accounts for the bilateral staining. But if unstained fibre is mechanically disrupted into cortical cells, is the structure which accounts for bilateral staining (in stained fibre) removed? If it is removed, then the residual fibre, which remains after liberation of some cortical cells, should show no bilateral staining if it is then stained by the method of Laxer and Ross.

This matter was investigated as follows, and the results are summarised in Table III-5. Residues of fibres ultrasonicated in 99% formic acid for 5 minutes and 1 hour showed bilateral staining in the light microscope when stained with gold. This differentiation was visible

both along the length of the fibre and in sections cut with a Hardy microtome. The fibre residue stained after ultrasonication of 1 hour was quite battered and the gold deposit was particularly heavy, owing to the advanced state of disruption; yet bilateral staining was still very obvious. To prevent misinterpretation of the results owing to the inherent variability of bilateral staining from fibre to fibre, a statistical count was made of the proportion of fibre cross-sections showing differentiation, for the two samples of stained and ultrasonicated fibre, and for fibre which was not ultrasonicated before staining. There was no decrease in the proportion of differentiated cross-sections in the ultrasonicated fibres. Fibres agitated in formic acid for 9 hours in a Vibromixer also showed differentiation along their length when subsequently gold stained. Hence the structure which accounts for the bilateral staining (in stained fibre) is not removed when unstained fibre is mechanically disrupted into cortical cells.

Cortical cells liberated from unstained wool by mechanical disruption were stained with 2% gold chloride in the usual manner and layered on a carbon tetrachloride/ethanol density gradient. A layer and a sediment were obtained, though each was considerably denser and more heavily stained than ortho- and paracortical cells separated from gold stained fibre. This is again evidence that the structure responsible for bilateral staining remains after the disruption of the fibre, though because of the large amount of gold deposited, some damage must have occurred, possibly on the surface of the cells.

Both ultrasonication in formic acid for 1 hour, and agitation in formic acid in the Vibromixer for 9 hours removes cuticle; yet the residual fibres when stained still showed preferential deposition in the paracortex; therefore the cuticle is not responsible for the bilateral staining. This is in agreement with results of earlier workers who obtained preferential staining of the orthocortex in fibre cross-sections post-stained with organic

dyes (in penetrating a cross-section a dye molecule can pass straight into the cortex without having to pass through a cuticle).

Wool stained with gold by the method of Laxer and Ross was examined in o-dichlorobenzene in the light microscope. The birefringence of the fibre was minimised by the solvent and the deposition of gold was plainly revealed. A dark red-brown colouration of the paracortex was visible, and dark, heavily stained streaks of about the same length as nuclear remnants were clearly delineated in that segment also. These streaks were not visible in fibres mounted in liquids which do not remove birefringence.

Ortho- and paracortical cell fractions prepared as in Section 3(a) from wool stained by the method of Laxer and Ross, were examined in o-dichlorobenzene in the light microscope. In each fraction cells having three types of staining were distinguished, but the proportion of the three types was different in the two fractions.

Type A: No dark streaks visible; either some or no pale red-brown colouration evenly distributed throughout the cell.

Type B: A single dark streak in the region of the nuclear remnant; a medium red-brown colouration throughout the rest of the cell.

Type C: A number of dark streaks both inside and on the surface of the cell; a medium red-brown colouration throughout the rest of the cell.

A statistical count on hundreds of cells from the ortho- and paracortical cell fractions showed the following distribution of the three types in the two fractions.

	Type A	Type B	Type C
Orthocortical cells	51%	25%	24%
Paracortical cells	18%	18%	64%

During the preparation of the two fractions the paracortical fraction was formed as a much more diffuse band than the orthocortical fraction in the density gradient. Nevertheless the two bands were distinctly separated.

These results might suggest that the two cortical cell fractions were contaminated by each other and that deposition of gold in the nuclear remnants accounts for the higher density of the paracortical cells (contrary to what the water washing experiments indicate). But if cross-contamination occurred why would the two fractions sediment at different densities?

The best evidence against contamination is that the electron microscope proved that the purity of the cell fractions was better than 99%, orthocortical cells being distinguished by their characteristic delineation of macrofibrils. Furthermore, Kulkarni et al. (1971) obtained separation on a density gradient of gold stained (lighter) orthocortical cells and (heavier) paracortical cells which had no gold particles in their nuclear remnants (no formic acid was used in the staining procedure). Although prior to staining their orthocortical cells were less dense than their paracortical cells (probably owing to the tryptic digestion and ultrasonication used to prepare them), the density of both kinds of cells was substantially increased by the gold staining, and so the preferential deposition of histochemical gold must have been responsible for the separation achieved of their gold stained cells. Hence in the work of this thesis, the preferential deposition of gold not in the nuclear remnants could account for the separation achieved, even though the remnants did contain gold particles.

Finally cross-section of ortho- and paracortical cells published by Kulkarni et al. show substantial removal of inter-macrofibrillar material, especially in the orthocortical cells. No such removal was seen in the cells prepared by this worker. Hence their unstained ortho- and paracortical cells have different densities, whereas cells liberated from unstained fibre by the mechanically disruptive methods developed in this laboratory cannot be separated on a density gradient into ortho- and paracortical cells of differing density (Chapman and Bradbury, 1968).

TABLE III-2

COMPARISON OF ELECTRON MICROGRAPHS

(Figs. III-4, III-5, III-6, III-7)

Formic Acid Series	Background colour	Distribution of small gold particles	Size and distribution of large gold particles in nuclear remnants
Fig. III-4	Darker in Paracortex	Occur more often in Paracortex	Are larger and occur more often in Paracortex
Fig. III-5	Darker in Paracortex	Occur more often in Paracortex	Remnants are not as full of particles as remnants in Fig. III-4. Also particles are smaller than in Fig. III-4
Fig. III-6	Darker in Paracortex	Particles are distributed almost evenly over both segments	Remnants of both segments are empty
Fig. III-7	Darker in Orthocortex	No particles visible	No particles visible. Remnants of Paracortex are stained deeply and evenly. Degradation visible in Paracortex

S.B.

"Formic acid series": Each sample from this series had the following treatment: (1) 2% AuCl_3 for time t. (2) 25% HCO_2H 4 hr.

"Water washed series": Each sample from this series had the following treatment: (1) 2% AuCl_3 for time t. (2) H_2O wash (several changes) 2 wk.

Fig. III-4: (1) 99% HCO_2H 20 min (2) 2% AuCl_3 pH 1.37 10 min
(3) 25% HCO_2H 4 hr

All samples were washed for 2 min, immediately after immersion in gold chloride, to remove the solution from the fibre surface.

Fig. III-5: (1) 2% AuCl_3 pH 1.37 10 min (2) 25% HCO_2H 4 hr

Fig. III-6: (1) 2% AuCl_3 pH 1.37 10 min (2) H_2O wash (several changes) 2 wk
(3) 25% HCO_2H 4 hr

Fig. III-7: Only 2% AuCl_3 pH 4.5 2½ months (Sample supplied by Mr. D.E. Peters)

TABLE III-3

EFFECT OF TIME OF GOLD CHLORIDE IMMERSION ON SAMPLES
FROM "FORMIC ACID SERIES" AND "WATER WASHED SERIES"

Formic Acid Series	Water Washed Series	Time t in 2% AuCl_3	Colour to eye	Colour in light m'scope	Bilateral staining
Yes	-	5-40 min	Dark purple	Dark red- brown	Yes
Yes	-	1½-36 hr	Pale rose- purple	Apparently colourless	No
-	Yes	5 min - 36 hr	Rose- purple	Pale red- brown	No

N.B.

"Formic acid series": Each sample from this series had the following treatment: (1) 2% AuCl_3 for time t (2) 25% HCO_2H 4 hr.

"Water washed series": Each sample from this series had the following treatment: (1) 2% AuCl_3 for time t (2) H_2O wash (several changes) 2 wk.

All samples were rinsed in water for 2 min, immediately after immersion in gold chloride, to remove this solution from the fibre surface.

TABLE III-4

ASH CONTENTS OF SAMPLES PRODUCED FROM WOOL BY STAINING WITH 2% GOLD CHLORIDE FOR 40 MINUTES

Treatment of fibre	Appearance of surface in light microscope	% of cross-sections showing bilateral staining
MERINO 64's		
Add 2% AuCl ₃ 40 min, then rinse in H ₂ O 2 min		
Gives A (Ash = 10.8%)		
Fibre ultrasonicated 5 min in 99% HCO ₂ H then gold stained		
Bilateral staining No disruption visible		
Water wash 2 wk	25% HCO ₂ H 4 hr	0.05 M H ₂ SO ₄ 4 hr
Fibre ultrasonicated 1 hr in 99% HCO ₂ H then gold stained	Bilateral staining Gold deposit heavy because fibre is battered	9%
B (Ash = 10.2%)	D (Ash = 10.3%)	E (Ash = 7.5%)
H ₂ SO ₄ 4 hr*		
C (Ash = 7.4%)	Bilateral staining No disruption visible	Not done
9 hr in 99% HCO ₂ H then gold stained		

* Actually three separate treatments with 5M, M, and 0.05M H₂SO₄ were used. The ash contents of the three resulting samples were within experimental error and so were averaged to give 7.4%.

TABLE III-5

EFFECT OF MECHANICAL DISRUPTION ON BILATERAL DIFFERENTIATION

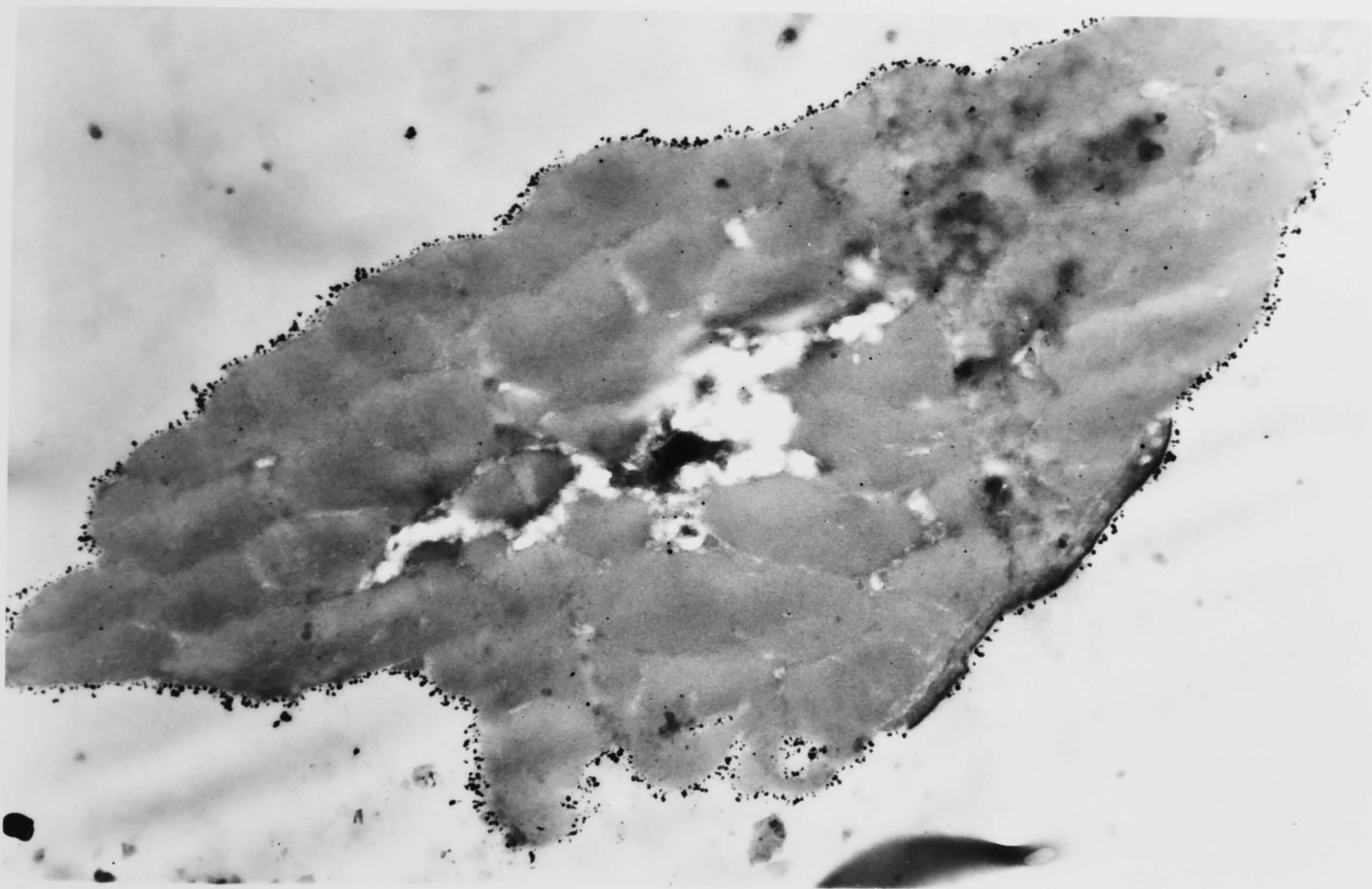
Treatment of fibre	Appearance of cortex in light microscope	% of cross-sections showing bilateral staining
Gold staining of undamaged fibre	Bilateral staining	85%
Fibre ultrasonicated 5 min in 99% HCO_2H then gold stained	Bilateral staining No disruption visible	71%
Fibre ultrasonicated 1 hr in 99% HCO_2H then gold stained	Bilateral staining Gold deposit heavy because fibre is battered	91%
Fibre Vibromixed 9 hr in 99% HCO_2H then gold stained	Bilateral staining No disruption visible	Not done

N.B. The method of Laxer and Ross (1954) was used to stain these samples.

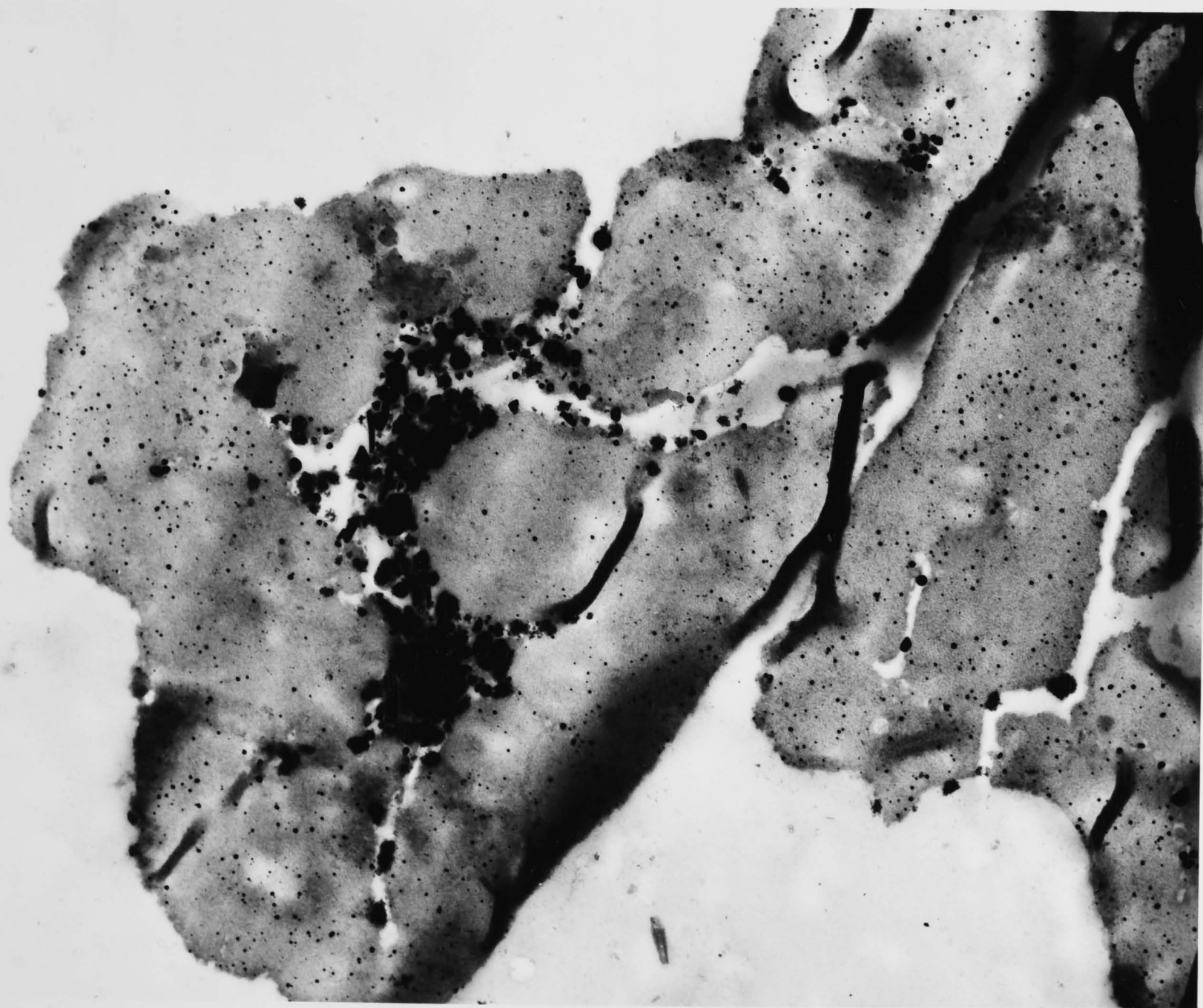
CAPTIONS TO MICROGRAPHS

Figure III-1: Cross-section of a gold stained cortical cell from the light fraction of cortical cells obtained from wool fibre bilaterally stained with gold. The delineation of macrofibrils proves that the cell is an orthocortical cell.

Figure III-2: Cross-section of a gold stained cortical cell from the heavy fraction of cortical cells obtained from wool fibre bilaterally stained with gold. No domains of macrofibrils are visible. Hence the cell is a paracortical cell.



III-1



III-2

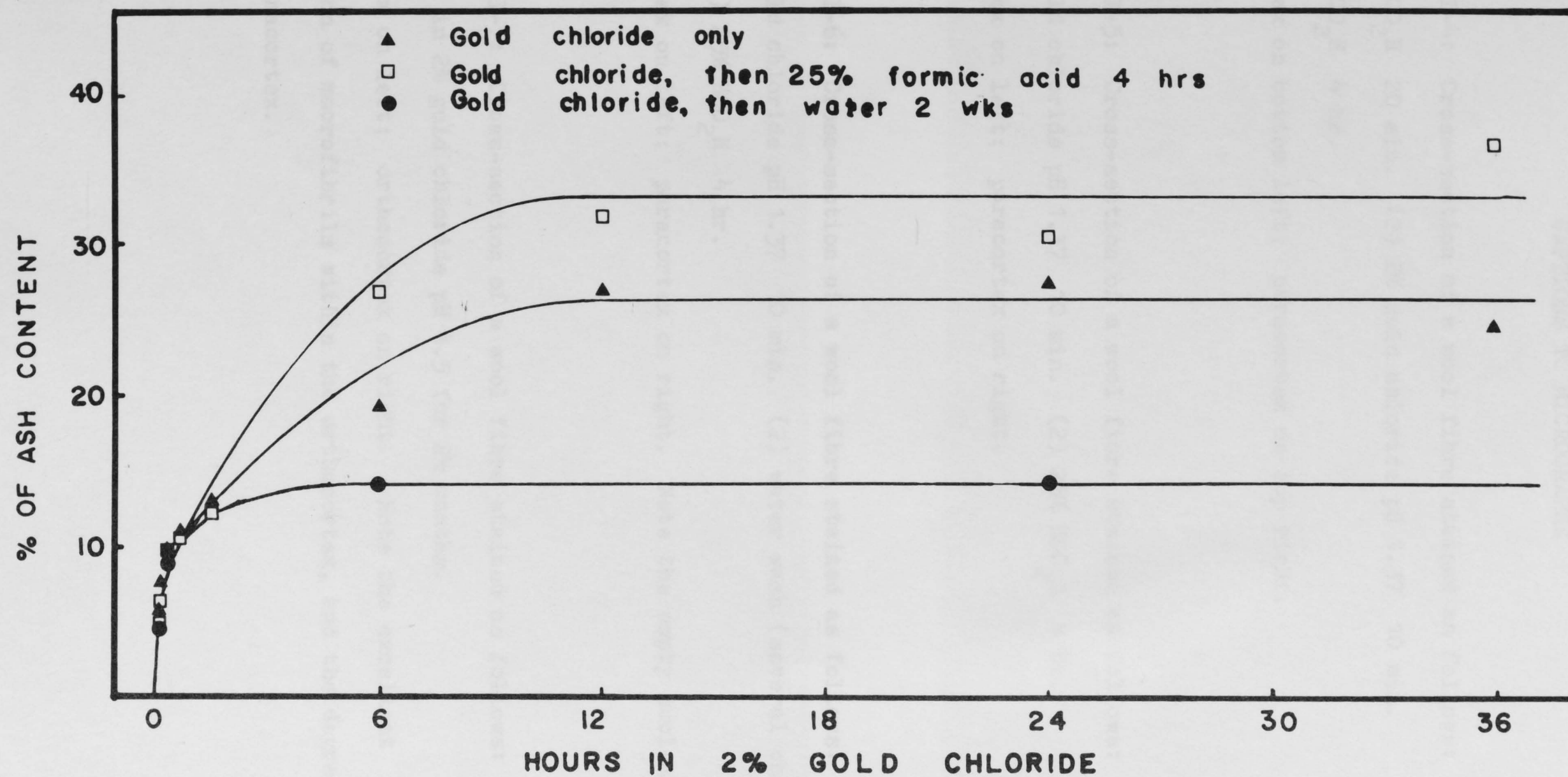


FIG. III-3. DEPENDENCE OF ASH CONTENT OF SAMPLES ON TIME IN GOLD CHLORIDE

CAPTIONS TO MICROGRAPHS

Figure III-4: Cross-section of a wool fibre stained as follows:

- (1) 99% HCO_2H 20 min. (2) 2% gold chloride pH 1.37 10 min.
(3) 25% HCO_2H 4 hr.

Orthocortex on bottom left; paracortex on top right.

Figure III-5: Cross-section of a wool fibre stained as follows:

- (1) 2% gold chloride pH 1.37 10 min. (2) 25% HCO_2H 4 hr.

Orthocortex on left; paracortex on right.

Figure III-6: Cross-section of a wool fibre stained as follows:

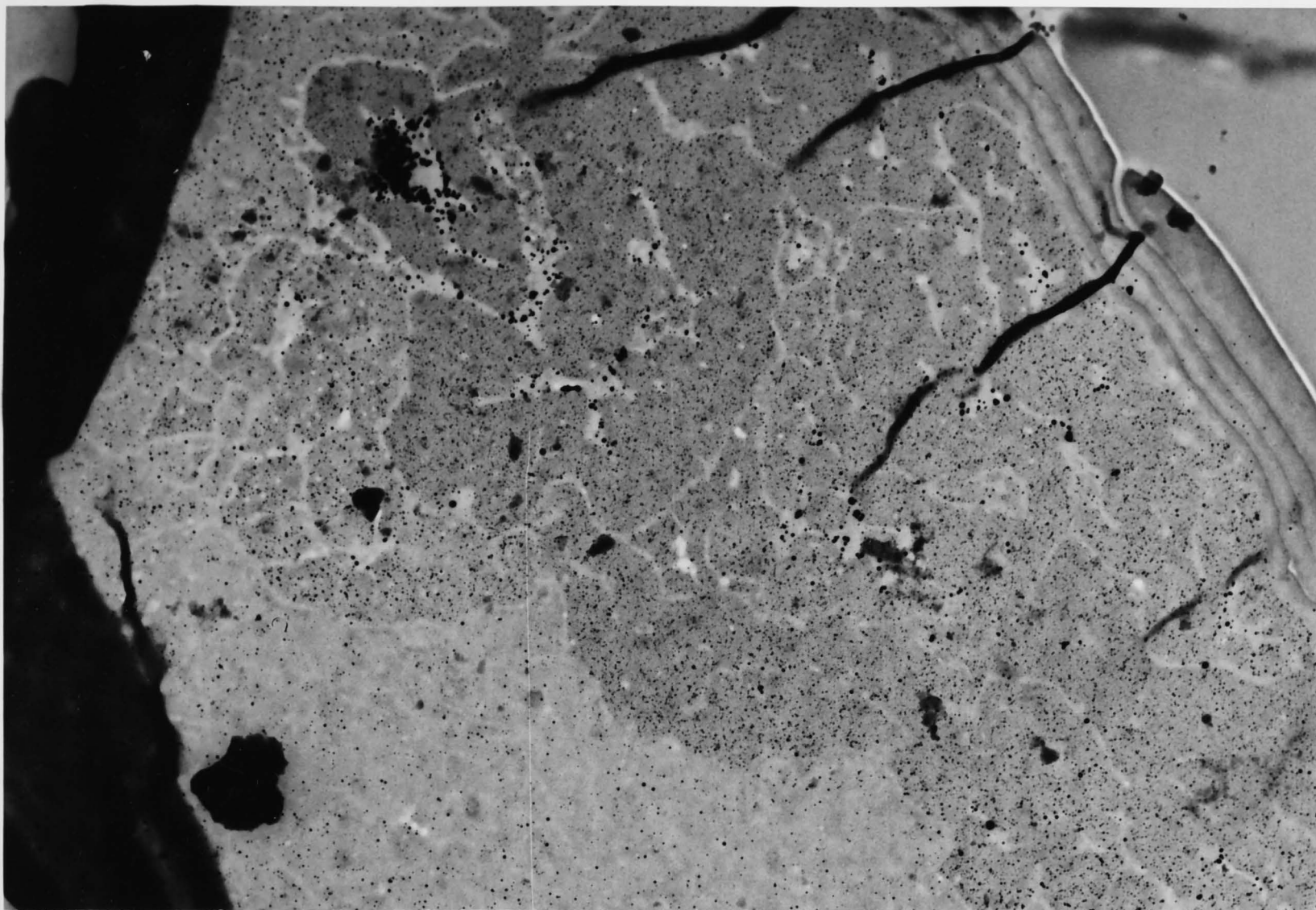
- (1) 2% gold chloride pH 1.37 10 min. (2) water wash (several changes)
2 wk. (3) 25% HCO_2H 4 hr.

Orthocortex on left; paracortex on right. Note the empty nuclear remnants.

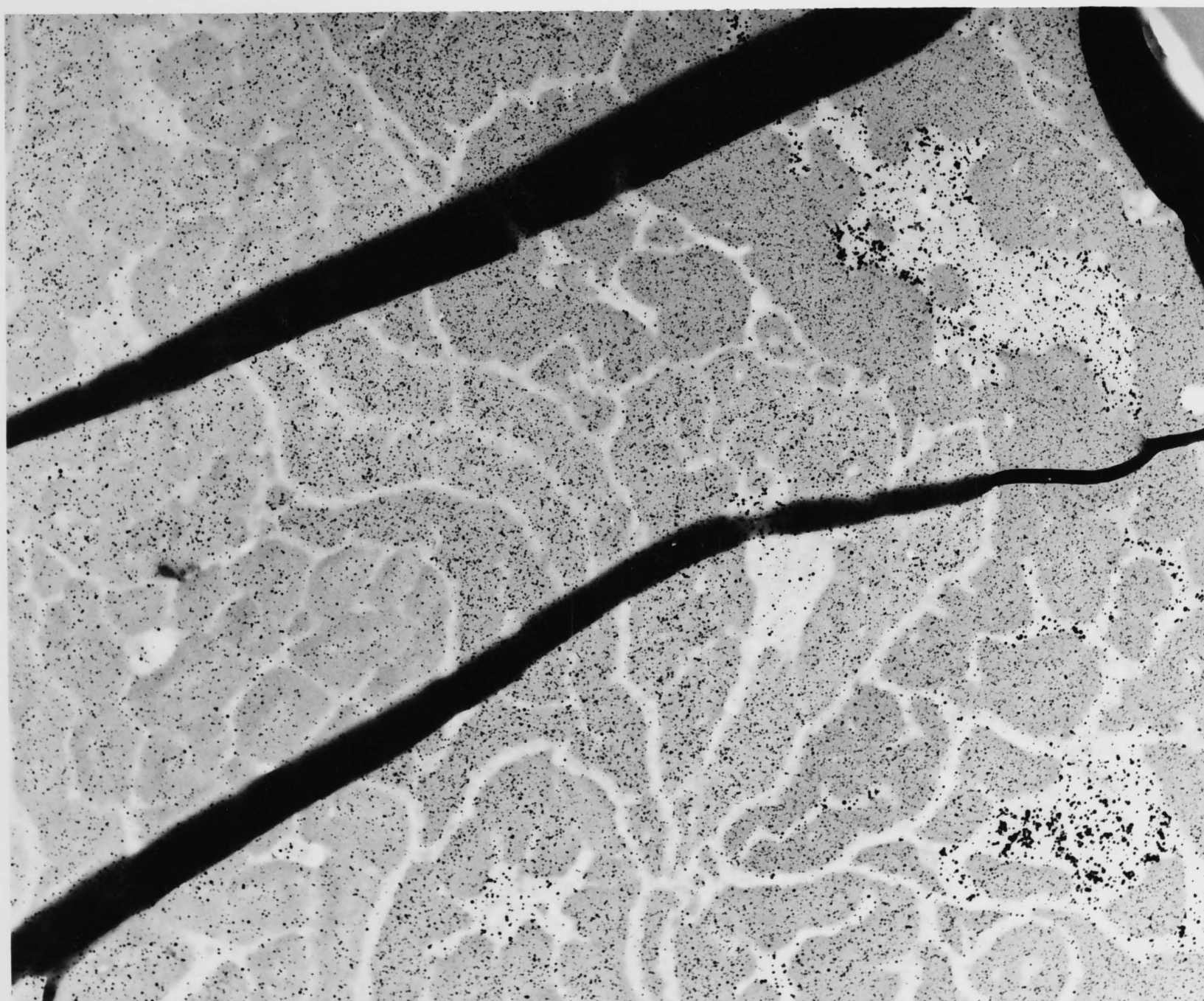
Figure III-7: Cross-section of a wool fibre stained as follows:

Immersion in 2% gold chloride pH 4.5 for 2½ months.

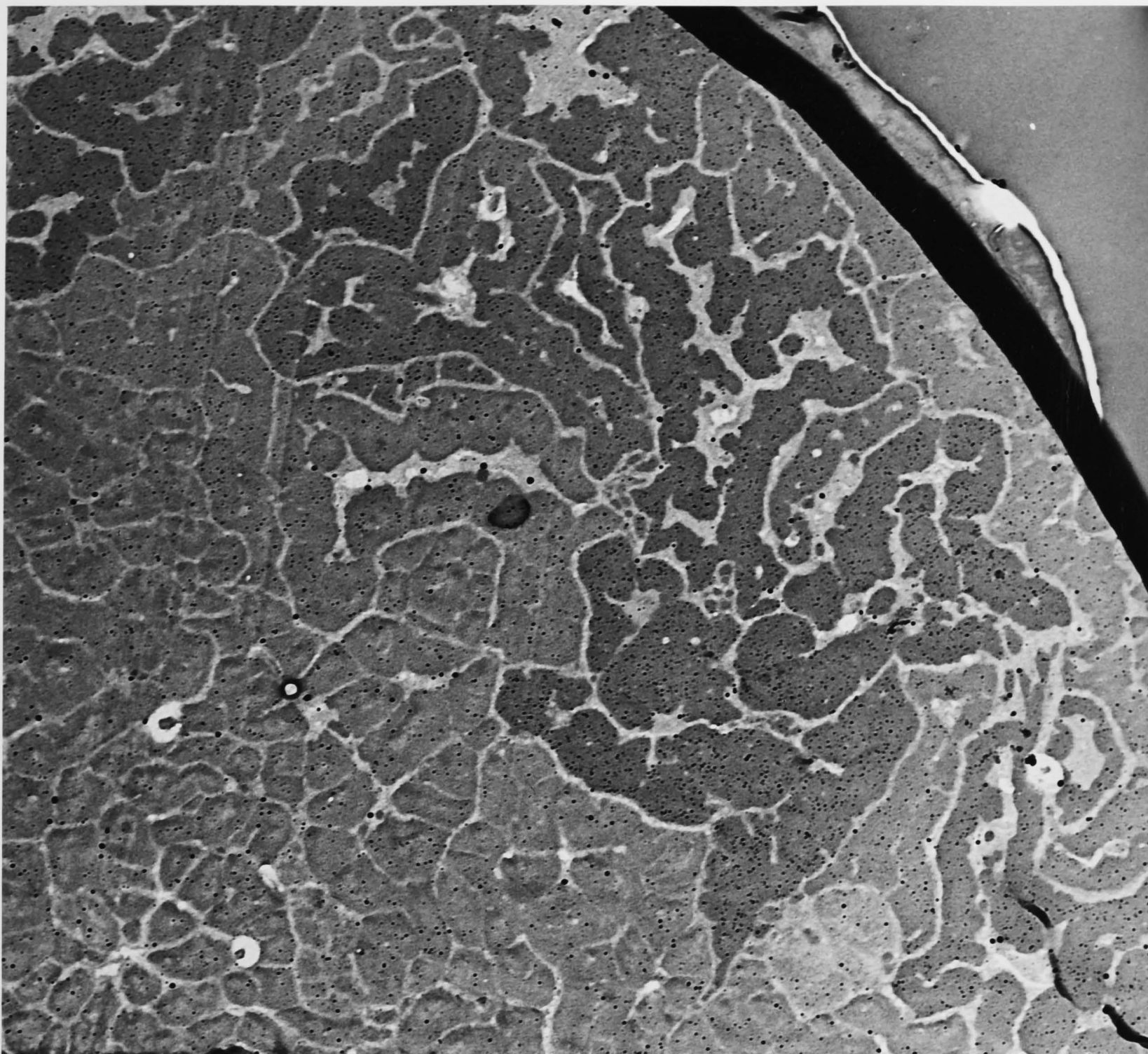
Paracortex on left; orthocortex on right. Note the excellent delineation of macrofibrils within the orthocortex, and the degradation in the paracortex.



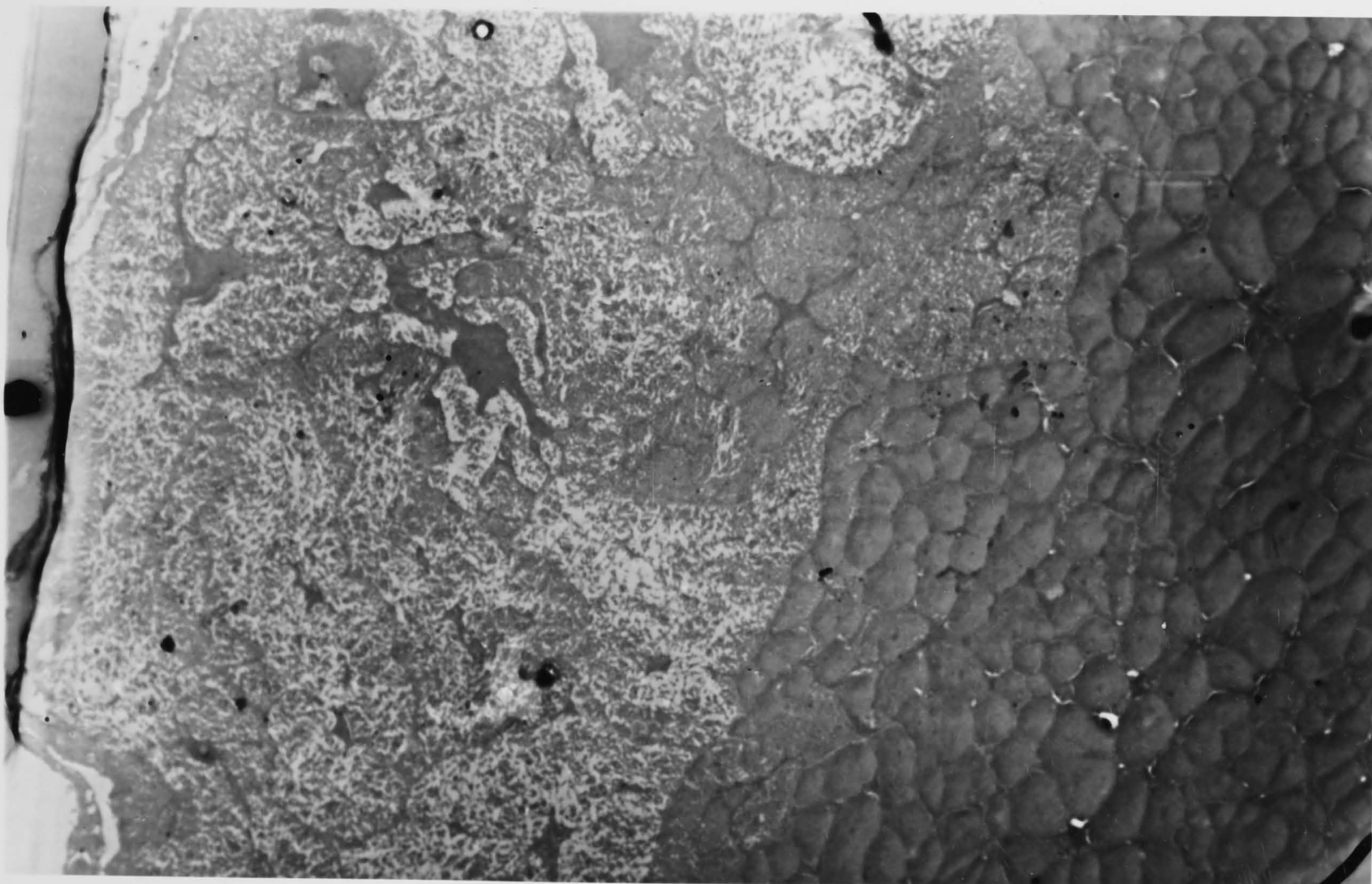
III-4



III-5



III-6



III-7

A P P E N D I X

An Analytical Method for the Determination of Gold in Gold Stained Wool Fibre and Cortical Cells.

The method involved converting all the gold in the gold stained keratin to bromoaurate ion - AuBr_4^- , and then measuring the absorption at 379 millimicrons of a solution of this ion. From a calibration curve of absorption of bromoaurate ion vs. concentration of gold, the amount of gold corresponding to the measured absorption can be determined.

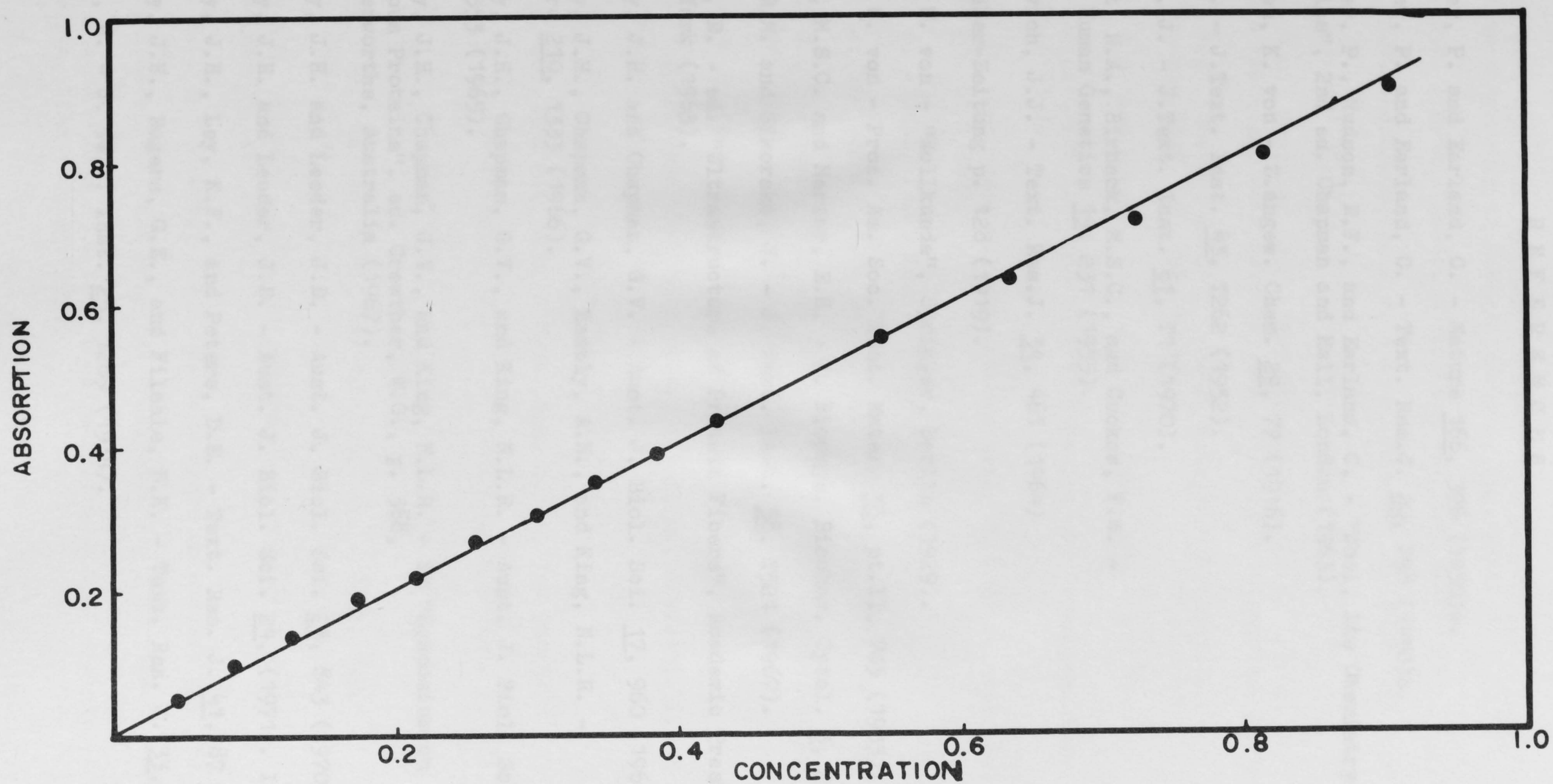
It was proposed to solubilise completely the gold stained keratin by hydrolysis, and then to convert all the gold in the solution (present either as colloidal gold or as some complex species) to some ion whose absorption could be measured. Preliminary studies were conducted of ultraviolet spectra of the following solutions, both when they contained solubilised gold and when they contained solubilised gold stained wool: HCl/HNO_3 ; $\text{H}_2\text{O}/\text{Br}_2$; HBr/Br_2 ; HBr/HNO_3 . Bromine was evaporated from the last three solutions before their spectra were obtained. Absorption bands at the wavelengths indicated were found for the following compounds (wavelengths in millimicrons): hydrochloric acid - below 250; chloroaurate ion - 311.5; nitric acid - below 250 and 303; bromine - 265 and 385; hydrobromic acid - below 250; bromoaurate ion - 254 and 379. After consideration of the interference possible between these bands, and of certain technical difficulties related to the solubilisation of the keratin, it was decided that the only suitable band was that at 379 millimicrons due to bromoaurate ion, provided bromine was completely evaporated off so as to remove the band at 385 millimicrons.

The accompanying calibration graph of absorption of bromoaurate ion at 379 millimicrons vs. concentration of gold, shows excellent linearity and so indicates that the Beer-Lambert Law holds for this absorption.

The graph was prepared by measuring the absorption at 379 millimicrons of solutions containing known amounts of gold dissolved in mixtures of hydrobromic and nitric acid.

The perfected method for determining gold is now presented. The determination should be performed in triplicate.

About 50 mg of the gold stained sample, previously dried in vacuo at 100°C , is immediately weighed quickly and accurately and then placed in a 25 ml round-bottomed flask. 10 ml of 48-50% w/w HBr is added to the flask, which is then fitted with a water condenser and refluxed gently for 3 hours or until no further dissolution of the sample occurs. Golden particles of gold can be seen on the bottom of the flask and the solution is coloured red with colloidal gold at this stage. Then 3.3 ml of concentrated HNO_3 are added slowly in small portions. A vigorous reaction which has an initiation period of some seconds occurs between the HNO_3 and the HBr to form Br_2 , and so great care must be taken to ensure that this reaction has subsided before the addition of each following portion of nitric acid. The mixture is very gently refluxed for 1 hour. All the gold dissolves in this time and so the condenser is then removed and all the bromine is very gently evaporated off, the level of the liquid being topped up with water during the evaporation. The liquid is then made up to 100 ml in a volumetric flask and the absorption at 379 millimicrons is measured. A control sample of the same weight of keratin, which is not stained with gold, is then subjected to an identical procedure, and the absorption of the resultant solution is measured; the value will be quite small. The absorption of the control is subtracted from that of the gold stained sample, and by use of the calibration curve and the original weight of the gold stained sample, the percentage of gold in that sample is calculated.



GRAPH OF ABSORPTION AT 379mμ VS. CONCENTRATION OF GOLD (mg / 25ml) IN GOLD CHLORIDE SOLUTION

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Separation of Orthocortical and Paracortical Cells from Wool

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KEYWORDS

Wool. Orthocortex; paracortex; cortical cell alignment; macrofibrils. Gold staining; electron microscopy; ultrasonication.

A method was developed for the separation of the orthocortex and paracortex of Merino wool [3, 5], which consisted of the preferential staining of the paracortex with gold [6], followed by disruption of the fiber by ultrasonication in formic acid. A clean sample of cortical cells was obtained by a differential screening procedure [2] and the heavily stained, dark red, paracortical cells were separated from the lightly stained, pink, orthocortical cells by centrifugation in a density gradient [5]. The amino acid analysis and content of high-sulfur and low-sulfur proteins from each fraction has been reported earlier [3, 5].

The gold was found by light microscopy to be located preferentially in the paracortex, and electron microscopy of cross sections stained with potassium permanganate showed that the metallic gold particles were concentrated in the nuclear remnants of the paracortex. On disruption of these fibers by ultrasonication in formic acid, a considerable amount of colloidal gold was liberated in the formic acid, but the residual fibers still retained their bilaterally stained appearance. We concluded that the colloidal gold which was liberated came from both orthocortex and paracortex and, hence, the heavily stained cortical cells did, in fact, originate from the paracortex. In order to make an unequivocal check of the correctness of this conclusion we have examined the heavily stained and lightly stained cortical cells by electron microscopy.

Merino 64's fibers were stained with gold, disrupted by ultrasonication, and the lightly stained and heavily stained cortical cells were separated by the method in [5], with only slight modifications (Ley, K. F., unpublished results). The two fractions were stained by the thioglycollate-osmium tetroxide method [8] and aligned by stroking in viscous Araldite on two Perspex microscope slides. The alignment was checked by light microscopy and the Araldite allowed to set hard. That part of each slide which contained the aligned cells was embedded in a block and sectioned perpendicularly to the cells, using an L.K.B. Ultratome. The sections were post-stained

with lead citrate [7] and examined in a Hitachi Model HU-11C-S electron microscope.

Figures 1 and 2 show cross sections of cortical cells from the light and heavy fractions, respectively, which are representative of the cells in these fractions. The cortical cell in Figure 1 originates from the orthocortex as shown by the typical appearance of the small, individual macrofibrils [1, 8]. The cortical cell in Figure 2 is not subdivided into macrofibrils as is an orthocortical cell, the nuclear remnant is large and filled with gold particles [5] and the microfibril-matrix structure is visible to a greater degree than in Figure 1. It is thus readily identified as a paracortical cell [8]. Approximately 800 cell sections from the light and heavy fractions were examined and in greater than 99% of cases the light fraction consisted

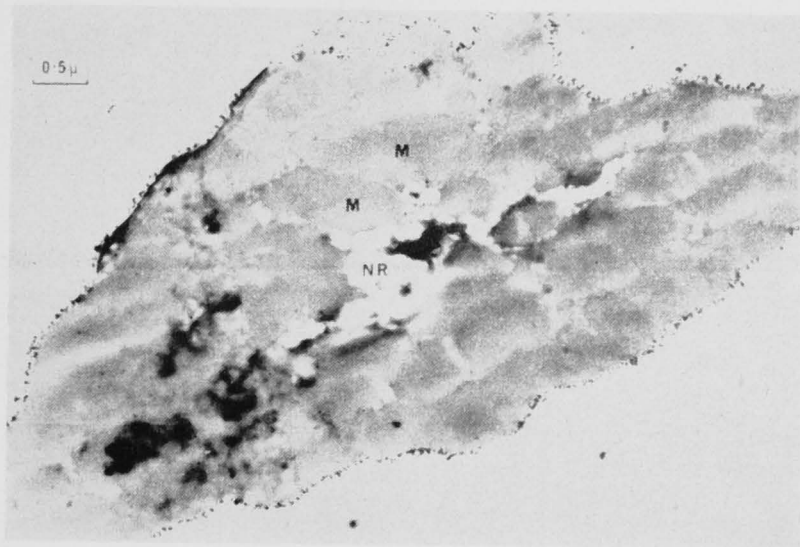


FIG. 1. Cross section of a cortical cell containing gold and originating from the light fraction. The clearly defined, macrofibrillar boundaries M show that this cell originates from the orthocortex [1, 8] in confirmation of earlier work [5].

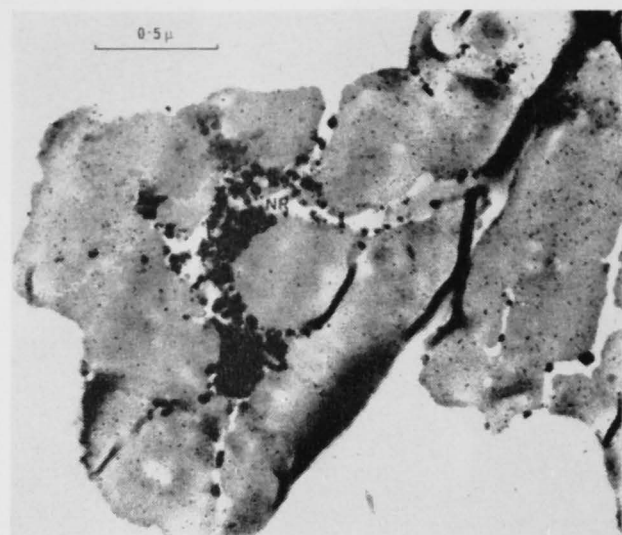


FIG. 2. Cross section of a cortical cell containing gold and originating from the heavy fraction. In confirmation of earlier work [5], this is identified as a paracortical cell on the basis of the absence of small circular macrofibrillar boundaries and the occurrence of large nuclear remnants NR in which metallic gold is concentrated [5].

of orthocortical cells and the heavy fraction, of paracortical cells. If a small percentage of mesocortical cells [1] occurs in Merino 64's wool, their fate would be unknown, since they may have a different density from either of the two main fractions or may occur in the heavy fraction with the paracortical cells.

These results provide proof of the separation of orthocortical and paracortical cells of fine wool [5]. The way in which the Au(III) is bound to the protein and the mechanism of deposition of gold metal is the subject of a further investigation [4].

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